Time-Resolved Immunofluorometric Dual-Label Assay for Simultaneous Detection of Autoantibodies to GAD65 and IA-2 in Children with Type 1 Diabetes

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Background: Autoantibodies to glutamic acid decarboxylase (GADAs), specifically the 65-kDa isoform GAD65, and autoantibodies to the protein tyrosine phosphatase-like molecule IA-2 (IA-2As) predict development of diabetes. Our aim was to develop a time-resolved immunofluorometric (TR-IFMA) dual-label assay method for the simultaneous detection of these autoantibodies and to evaluate the diagnostic sensitivity of the method compared with single-label TR-IFMA and fluid-phase radiobinding assay (RBA) in screening children with type 1 diabetes.

Methods: We combined biotinylated GAD65 and IA-2 proteins, glutathione S-transferase (GST)-IA-2, europium-labeled GAD65, terbium-labeled anti-GST antibody, and serum sample or calibrator and transferred aliquots to a streptavidin-coated 96-well microtiter plate for incubation. After washing, we added Delfia Enhancement solution to each well and measured the fluorescence of Eu. We developed the Tb fluorescence signal by use of the Delfia Enhancer solution and measured it. We analyzed serum samples from a cohort of 100 children with newly diagnosed type 1 diabetes.

Results: The correlation coefficients between the autoantibody concentrations measured by dual- and single-label TR-IFMA assays were 0.962 for GADA and 0.874 for IA-2A. Among 100 children with newly diagnosed diabetes, 65 of them were GADA positive in the dual-label assay, 64 in the single-label assay, and 66 in the RBA GADA assay. Seventy-four of the children tested positive for IA-2A in both TR-IFMA assay types, and 79 in the RBA IA-2A assay.

Conclusions: The novel dual-label immunofluorometric assay performed comparably to the separate, single-label GADA and IA-2A assays in screening for β-cell autoimmunity in children with newly diagnosed type 1 diabetes.

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Type 1 diabetes mellitus results from an autoimmune process that destroys the insulin-producing β-cells of the pancreatic islets. The appearance of autoantibodies to β-cell antigens, such as insulin, the 65-kDa isoform of glutamic acid decarboxylase (GAD65), and protein tyrosine phosphatase-like molecule (IA-2), into the peripheral circulation predicts clinical disease, and the presence of multiple autoantibodies and IA-2 autoantibodies (IA-2As) indicates high probability that the disease will appear soon (1, 2). Autoantibody measurements can also be used to help differentiate between late-onset autoimmune diabetes and classic type 2 diabetes (3, 4). Each specific autoantibody alone has relatively poor sensitivity and

* Nonstandard abbreviations: GAD65, 65-kDa isoform of glutamic acid decarboxylase; IA-2, protein tyrosine phosphatase-like molecule; IA-2A, autoantibody to IA-2; GADA, autoantibody to glutamic acid decarboxylase; RBA, radiobinding assay; TR-IFMA, time-resolved immunofluorometric assay; and GST, glutathione S-transferase.
specificity for disease prediction; accordingly, there is a need to measure several of them simultaneously. Identifying the combination of autoantibodies to glutamic acid decarboxylase (GADAs) and IA-2As is useful in risk estimation—a vast majority of patients with type 1 diabetes have at least one of these autoantibodies, and the presence of both indicates a high disease risk in unaffected individuals.

Several methods have been developed for the detection of GAD65 and IA-2 autoantibodies; some assays are available commercially. The radiobinding assay (RBA), which has proven reliable and is widely used (5–8), is based on in vitro production of the antigen in the presence of a radioactive label, usually $[^{35}S]$methionine, and capture by protein A Sepharose of the immune complexes formed with serum antibodies. A modification of the standard RBA that combines both GADA and IA-2A measurements in 1 well has been developed for screening (9). Two RBA methods using GAD65–IA-2 fusion proteins for the same purposes have been published by us and another research group (10, 11). These tests cannot discern between GADA and IA-2A autoantibodies: further separate tests must be done in cases that screen positive. Disadvantages of the RBA include the need of handling radioactive isotopes, complicated reagent synthesis, and extended duration of the measurements. In addition, RBA formats in which gel beads are used to capture immune complexes do not allow automation at the efficiency that solid-phase methods do.

We previously published single-label time-resolved immunofluorometric assay (TR-IFMA) methods for the detection of GADAs and IA-2As in a solid-phase streptavidin-coated 96-well microtiter plate format (12, 13). The detection uses europium lanthanide chelate as a fluorescent label. In this report, we describe the development of a rapid and convenient immunofluorometric dual-label assay for the simultaneous analysis of GADAs and IA-2As based on time-resolved fluorescence of Eu and terbium chelates, and we compare the experimental data to that obtained with the single-label TR-IFMA and RBA assays.

**Materials and Methods**

**REAGENTS AND INSTRUMENTATION**

We purchased the recombinant human GAD 65-kDa isoform protein, GAD65, from Diamyd Diagnostics AB and stored it in aliquots at −70 °C until use. The protein was expressed in Spodoptera frugiperda (Sf9) insect cells via baculoviral infection. IA-2 and glutathione S-transferase (GST)–IA-2 proteins were produced in Escherichia coli BL21 cells and purified as described previously (13).

Biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester was a product of Sigma. Streptavidin-coated microtiter plates, DTTA (N1,N2,N3,N4-tetraacetic acid)–Eu chelate, DTTA-Tb chelate, Diluent II, Delfia Assay Buffer, Wash Solution, Enhancement Solution, Delfia Enhancer, and Eu and Tb standard solutions were obtained from PerkinElmer Life and Analytical Sciences Wallac Oy. Polyclonal goat anti-GST antibody, NAP-5, and Superdex 200 gel filtration columns were purchased from GE Healthcare Amersham Biosciences AB. The monoclonal anti-GAD65 antibody MAB351R was a product of Chemicon International, and the monoclonal human anti-IA-2 antibody (clone 96/3) was produced by Roche Diagnostics. Delfia 1234 Plate Fluorometer, Delfia Plate Shake, and Delfia Plate Wash were products of PerkinElmer Life and Analytical Sciences Wallac Oy.

**SAMPLE AND CALIBRATION MATERIAL**

We analyzed serum samples from 100 children and adolescents taken at the diagnosis of type 1 diabetes (56 boys, mean age 11.7 years, range 7.2–16.0 years) and 100 healthy controls (56 boys, mean age 11.8 years, range 7.2–16.0 years).

For calibration, we used a monoclonal anti-GAD65 antibody, MAB351R, and a monoclonal human anti-IA-2 antibody, clone 96/3. We prepared the calibrator series by diluting the antibodies in a GADA- and IA-2A–negative serum pool (dual-label assay) or Diluent II containing 50 mmol/L Tris-HCl, 9 g/L NaCl, 0.5 g/L sodium azide, and 75 g/L bovine serum albumin, pH 7.8 (single-label assays).

**BIOTINYLATION OF GAD65 AND IA-2**

Biotinylation was performed at pH 7.4 in 50 mmol/L HEPES, 9 g/L NaCl solution at room temperature for 4 h with the use of 30-fold (GAD65) or 50-fold (IA-2) molar excess of biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester. We separated the biotinylated protein from the free biotinylation reagent by gel filtration on a NAP-5 column and used a buffer containing 50 mmol/L HEPES, 9 g/L NaCl, and 0.5 g/L NaN3, pH 7.4, for the elution.

**LABELING WITH LANTHANIDE CHELATES**

We performed Eu-labeling of GAD65 with a 50-fold molar excess of DTTA-Eu chelate in 50 mmol/L NaHCO3–Na2CO3, 9 g/L NaCl buffer, pH 9.3, at 4 °C for 16–20 h. We purified the Eu-labeled GAD65 by gel filtration on a NAP-5 column as with the biotinylated proteins. The molar labeling degree, the number of Eu chelates covalently bound per 1 GAD65 molecule, was 10–17 when measured against a Eu standard solution.

Polyclonal goat anti-GST antibody was labeled at 4 °C for 18 h with a 50-fold molar excess of DTTA-Eu (for single-label IA-2A assay) or DTTA-Tb chelate in a 100 mmol/L (Eu labeling) or 50 mmol/L (Tb labeling) NaHCO3–Na2CO3, 9 g/L NaCl buffer, pH 9.3. We purified the Tb-labeled anti-GST antibody by gel filtration on a NAP-5 column as above, but used a Superdex 200 gel filtration column for the purification of Eu-labeled anti-GST antibody. The labeling degree was typically 8 for the anti–GST-Eu and 10 for the anti–GST-Tb, when Eu and Tb standard solutions were used.
DUAL-LABEL ASSAY

We incubated biotinylated GAD65 and IA-2 proteins, GST-IA-2, Eu-labeled GAD65, Tb-labeled anti-GST antibody, serum sample or calibrator, and 180 μL Delfia Assay buffer plus 2 g/L casein (casein buffer) in 1.5-ml Eppendorf tubes in a total volume of 210 μL for 45 min with continuous shaking on a Delfia Plate Shake. We transferred 100-μL aliquots into 2 parallel wells on a streptavidin-coated 96-well microtiter plate and continued incubation for 30 min on the shaker. The aliquots corresponded to 75 ng biotinylated GAD65, 20 ng biotinylated IA-2, 25 ng GST–IA-2, 25 ng Eu-labeled GAD65, 15 ng Tb-labeled anti-GST, and 15 μL sample or calibrator per well. We washed the wells 4 times with Delfia Wash Solution using a Delfia Plate Wash and added 150 μL Delfia Enhancement solution to each well, and the plate was shaken for 15 min on a Plate Shake. We measured the fluorescence of Eu in a Delfia 1234 Plate Fluorometer and calculated the results by use of the Wallac Multicalc program. All samples were analyzed in duplicate.

In the IA-2A assay, we incubated 10 μL serum sample or calibrator for 60 min with 40 ng biotinylated IA-2 and 50 ng GST-IA-2 in 100 μL casein buffer. We performed 2 washing rounds, added 15 ng Eu-labeled anti-GST antibody in 100 μL casein buffer, and continued incubation for 30 min. We washed the wells 4 times, added 200 μL Delfia Enhancement solution, and measured Eu fluorescence as described above.

SINGLE-LABEL GADA AND IA-2A ASSAYS

We performed the single-label TR-IFMA assays at room temperature on streptavidin-coated 96-well microtiter plates as described by us for GADA (12) and IA-2A (13), with minor modifications. Briefly, in the GADA assay, we incubated 75 ng biotinylated GAD65 per well in 100 μL Delfia Assay buffer for 45 min with continuous shaking on a Plate Shake. We washed the wells once with Delfia Wash Solution using a Delfia Plate wash, added 50 μL Delfia Assay buffer and 10 μL serum sample or calibrator to each well, and continued incubation for 45 min with shaking. After 2 washing rounds, we added 25 ng Eu-labeled GAD65 in 75 μL Delfia Assay buffer and continued incubation for 30 min on a Plate Shake. We performed 4 washing rounds, added 100 μL Delfia Enhancement solution to each well, and continued incubation on a Plate Shake for 15 min. We measured the fluorescence in a Delfia 1234 Plate Fluorometer and calculated the results by use of the Wallac Multicalc program. All samples were analyzed in duplicate.

In the IA-2A assay, we incubated 10 μL serum sample or calibrator for 60 min with 40 ng biotinylated IA-2 and 50 ng GST-IA-2 in 100 μL casein buffer. We performed 2 washing rounds, added 15 ng Eu-labeled anti-GST antibody in 100 μL casein buffer, and continued incubation for 30 min. We washed the wells 4 times, added 200 μL Delfia Enhancement solution, and measured Eu fluorescence as described above.

GADA IGM ASSAY

We performed the TR-IFMA assay measuring IgM-class GADAs as a single-label GADA assay but used biotinylated antihuman IgM antibody (BD PharMingen) instead of biotinylated GAD65. We measured the patient and control samples giving discrepant GADA positivity in the TR-IFMA and RBA assays. Because no calibration method was available, the results were expressed semiquantitatively as negative or weak, moderate, or high positive activity, based on the signal level achieved. We compared the signal to the Eu signal measured from 10 negative samples in the Diabetes Autoantibody Standardization Program (DASP) 2005 sample panel.

RADIOBINDING ASSAYS

We performed all the RBA analyses in the Research Laboratory, Department of Paediatrics, University of Oulu, by use of modified RBAs as described previously (7, 8).

ASSAY OPTIMIZATION

During development of the dual assay, we tested a large series of different assay component concentration and reaction condition variables and their combinations and optimized them to achieve the best combination of sufficient signal level, linear response interval, signal-to-noise ratio, and analytical sensitivity, specificity, and reproducibility.

DUAL-LABEL ASSAY PERFORMANCE CHARACTERISTICS

The calibration curves for the single- and dual-label assays were linear over the whole calibration interval (0–1000 μg/L for GADA and 0–500 μg/L for IA-2A), and no high-dose hook effect was observed up to 3500 μg/L for GADA and 650 μg/L for IA-2A.

Reproducibility. The variation of the dual-label TR-IFMA assay was measured in 20 runs (4 replicates per run) using 4 serum samples that represented different, clinically
relevant concentrations of GADA and IA-2A. The mean intraassay, interassay, and total CVs are summarized in Table 1.

**Detection limit.** The detection limit (limit of the blank) of the dual assay was 0.8 μg/L for GADA and 0.3 μg/L for IA-2A, when defined as the signal + 3 SD detected from a negative serum.

**Linearity.** We serially diluted 4 patient serum samples in the interval of 52–1430 μg/L (GADA) and 65–570 μg/L (IA-2A) in a GADA- and IA-2A-negative serum pool. We measured the observed vs calculated GADA and IA-2A concentrations in 4 different dilutions, each dilution twice in 2 replicates. For GADA, the mean of all observed values was 103% of the calculated values, and the total range was 93%–122%. The mean results for each of the 4 sample dilution series were in the range of 97%–113%, and the linear regression coefficient (r) was 0.998 for all of the series. The mean of the observed values was 102% of the expected values for IA-2A, and the range was 91%–123%. For each of the 4 sample dilution series, the mean results were in the range of 96%–114%, and the linear regression coefficient (r) was 0.997 for all the series.

**Recovery.** To 3 serum samples containing a known amount of GADAs and IA-2As, we added 3 concentrations (20–400 μg/L) of monoclonal anti-GAD antibody MAB351R and 3 concentrations (10–200 μg/L) of monoclonal anti-human IA-2 antibody clone 96/3. The mean recoveries were 96% for GADA and 97% for IA-2A when 3 replicates were used in each assay. The recoveries ranged from 90% to 102% and from 89% to 107%, respectively.

**Clinical results**
The cutoff concentrations of the TR-IFMA assays were set at the mean + 3 SD in the present series of 100 healthy control children, i.e., at 13.2 μg/L for the single-label and 12.8 μg/L for the dual-label GADA assay. For the IA-2A assays, the cutoff limits were 1.14 μg/L and 1.57 μg/L for the single- and dual-label assays, respectively.

We measured 100 sera samples from children with newly diagnosed type 1 diabetes and 100 sera samples from healthy control children with the TR-IFMA single- and dual-label assays and RBA methods. Correlation was strong between the antibody concentrations obtained by the dual- and single-label TR-IFMA GADA and IA-2A assays. When all 200 patient and control samples were measured, the linear correlation coefficient (r) was 0.962 for GADA and 0.874 for IA-2A assays (Fig. 2). On the other hand, the results of the dual-label TR-IFMA assay and the RBA assays did not correlate well linearly, mainly because of the limited linear range of the RBA assays at high autoantibody concentrations, especially in the GADA assay, as can be seen from the shapes of the correlation graphs (Fig. 3).

Sixty-five of the 100 samples from children with type 1 diabetes were GADA-positive in the dual-label assay and 64 in the single-label TR-IFMA GADA assay. Both IFMA methods detected 62 of them. The RBA GADA assay detected 66 positive samples, and 2 of them were positive only in the RBA. Three samples were GADA-positive in both IFMA assays but negative in the RBA analysis. Both the dual- and single-label TR-IFMA assays detected 74 IA-2A–positive samples among the 100 samples from

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**Table 1. Reproducibility of the TR-IFMA GADA and IA-2A dual-label assay results.**

<table>
<thead>
<tr>
<th></th>
<th>GADA, %CV</th>
<th>IA-2A, %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraassay</td>
<td>5.6</td>
<td>9.1</td>
</tr>
<tr>
<td>Interassay</td>
<td>8.1</td>
<td>12</td>
</tr>
<tr>
<td>Total variation</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
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Fig. 2. Comparison of dual- and single-label TR-IFMA assays. The cutoff values for the 2 assays are indicated by dashed lines.
diabetic children, and 79 samples were detected positive in the RBA IA-2A assay, 74 of them positive in all assay types (Table 2). The few controversial samples had mainly low autoantibody concentrations, close to the cutoff limits. All the discrepant samples GADA-positive in the TR-IFMA assay also showed weak or moderate GADA IgM-positive activity (Table 3).

The autoantibody concentration at the onset of type 1 diabetes did not correlate with the age of the patient (Fig. 4). Of the samples from patients with type 1 diabetes under 12 years of age, 69.4% (34/49) were GADA-positive in the IFMA dual assay, as were 60.8% (31/51) of those from children older than 12 years; 73.5% (36/49) and 74.5% (38/51) were positive for IA-2As.

Among the 100 samples from control children, the single-label IFMA IA-2A assay detected 2 and the dual-label assay detected 1 IA-2A–positive sample, and 3 GADA-positive samples were detected in both single- and dual-label IFMA assays. The autoantibody concentrations were low in all the cases, near cutoff limits. All the GADA-positive control samples also had GADA IgM activity (data not shown). One control sample was positive for GADAs in dual-label and IA-2A–positive in single-label assay. All the control samples were negative in both RBA assays. Accordingly, the specificity of the dual-label TR-IFMA assay was 97% for GADA and 99% for IA-2A, and the specificities of the single-label assays were 97% and 98%, respectively. In this control cohort the specificity of both RBA assays was 100%.

In the latest DASP 2005 Workshop, the sensitivities for our single-label TR-IFMA GADA and IA-2A assays were 80% and 70%, and the specificities were 95% and 99%, respectively. The dual-label assay results were not reported to the DASP organization, but the sensitivities were 80% and 72% and specificities 94% and 99%, respectively.

Discussion
Autoantibodies to GAD65 and IA-2 are informative in the assessment of the risk for type 1 diabetes, and knowledge of autoantibody status is needed to identify individuals at risk, who are eligible for preventive trials and studies elucidating the mechanisms of the progressive autoimmune process. The need for large screening programs will arrive when effective treatments able to stop or delay the disease process are developed. High-throughput assay platforms and the possibility to combine GADA and IA-2A measurements into a single test will be of advantage. It has been demonstrated that the vast majority of children with type 1 diabetes test positive for at least 1 of these 2 autoantibodies at clinical presentation of the disease. Preliminary data also indicate that these antibodies are usually present months and years before the appearance of any clinical signs of type 1 diabetes (14, 15).

The patient group was randomly chosen among children with newly diagnosed type 1 diabetes and was not identical to the sample populations in our previous publications (12, 13). There were fewer individuals positive for GADAs and more for IA-2As in this panel than in
patient populations in general. The frequency of autoantibody positivity varies to some extent in relatively small patient cohorts. The number of GADA- and IA-2A–positive samples obtained with the RBAs in the present study correlated very well with the IFMA results. We have previously observed and reported that in the Finnish population the frequency of various autoantibodies depends on the age and HLA genotype of the participants. Thus IA-2As were more common than GADAs in young children, whereas GADAs were more frequent than IA-2As in individuals older than 20 years at the onset of type 1 diabetes (16). Multiple studies have also shown that IA-2As are associated with the HLA-DR4/DQ8 haplotype, whereas GADAs are associated with DR3/DQ2. The Finnish population is characterized by a high frequency of DR4/DQ8 compared with DR3/DQ2, whereas DR3/DQ2 is more common in populations from southern and central Europe.

The assay protocol, in which all the antigen-antibody interactions were allowed to occur during a combined incubation phase in a specific solution environment and the formed immunocomplexes were concentrated in a separate incubation step onto the solid-phase streptavidin via the biotin chemistry, worked well. The selected strategy is also simplest. In fact, our attempts to combine the individual single-label assays into 1 traditional stepwise assay procedure with 2 or 3 separate incubation steps did not result in satisfactory performance. Especially in IA-2A detection, the signal level was low and the sensitivity decreased considerably.

In general, the dual-label assay gave lower GADA and IA-2A concentrations than the single-label assays did, especially at high concentrations, probably because in the dual-label assay there are more biotin-labeled molecule complexes competing for binding to the streptavidin coating in the well. The numerical correlation between the concentrations provided by the dual- and single-label assays was usually good. Some cases, however, showed higher or lower concentrations in the dual-label assay. In the dual-label assay, the antigen and the antibody molecules react freely in solution conditions, whereas in the single-label assays the biotinylated antigen is immobilized on the well surface. The lower results in the dual-label assay may be explained by the autoantibodies bound to the biotinylated antigen, which sterically could limit the binding of the antigen to the streptavidin on the well surface. On the other hand, in the dual assay, the amounts of GST-IA-2 and biotinylated IA-2 were reduced to half and the sample volume increased from 10 μL to 15 μL compared with the single-label IA-2A assay. This compromise resulted in increased analytical sensitivity but decreased the linear range of the IA-2A assay. In cases where the dual-label assay gave higher concentrations, the liquid reaction conditions probably favor antibody binding to the free antigen in comparison to the surface-bound antigen.

In single-label assays, the calibrator series was diluted in Tris-buffer based Diluent II, which gave optimal sensitivity and low background signal. Usually the sera from autoantibody-negative young children gave lower background signals than the negative serum pools from adolescents or adults. Probably because of the properties of the Tb label and the Tb Delfia Enhancer solution, the background signal was high and the sensitivity poor in the dual-label assay IA-2A detection, if the Diluent II coated in the well. The numerical correlation between the concentrations provided by the dual- and single-label assays was usually good. Some cases, however, showed higher or lower concentrations in the dual-label assay. In the dual-label assay, the antigen and the antibody molecules react freely in solution conditions, whereas in the single-label assays the biotinylated antigen is immobilized on the well surface. The lower results in the dual-label assay may be explained by the autoantibodies bound to the biotinylated antigen, which sterically could limit the binding of the antigen to the streptavidin on the well surface. On the other hand, in the dual assay, the amounts of GST-IA-2 and biotinylated IA-2 were reduced to half and the sample volume increased from 10 μL to 15 μL compared with the single-label IA-2A assay. This compromise resulted in increased analytical sensitivity but decreased the linear range of the IA-2A assay. In cases where the dual-label assay gave higher concentrations, the liquid reaction conditions probably favor antibody binding to the free antigen in comparison to the surface-bound antigen.

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The novel dual-label immunofluorometric assay for GADA and IA-2A performed well, in comparison to the separate, single-label GADA and IA-2A TR-IFMAs and RBAs, when detecting signs of H9252-cell autoimmunity in children with type 1 diabetes. The setting of cutoff value is especially critical in the GADA assay, because there is no clear distribution between positive and negative GADA values (Fig. 4A). Patient and control samples having discrepant GADA positivity compared with the traditional RBA assay were shown to have IgM-class GADAs. IgM-specific autoantibody response is the initial response in prediabetic individuals (17). This result emphasizes the predictive value of our new method, which is simple and rapid to perform and is easily adaptable to an automated platform, allowing significant additional reduction in performance time and labor costs.

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References

