Evaluation of Two Nonisotopic Immunoassays for Determination of Glutamic Acid Decarboxylase and Tyrosine Phosphatase Autoantibodies in Serum

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Background: Autoantibodies for the 65-kDa form of glutamic acid decarboxylase (GAD65) and protein tyrosine phosphatase-like protein (IA-2) are measured for risk prediction and diagnosis of autoimmune diabetes mellitus. There is a lack of adequate nonisotopic alternatives to the most widely used method for both autoantibodies, which is a radiobinding assay (RBA).

Methods: We compared two commercially available immunoassays, an ELISA and a time-resolved immunofluorometric assay (TR-IFMA), with RBA.

Results: We found excellent agreement between the RBA and ELISA for measurement of GAD65 autoantibodies (GADAs); they showed comparable analytical precision in the cutoff range and achieved similar diagnostic specificity. The ELISA identified more GADA-positive individuals among patients with new-onset type 1 diabetes than did the RBA [89% (95% confidence interval, 78–95%), vs 71% (58–82%); P <0.03]. For IA-2 autoantibodies (IA-2As), only the TR-IFMA achieved analytical performance and diagnostic accuracy comparable to that of the RBA. These results with the GADA ELISA and IA-2A TR-IFMA were consistent with those obtained blindly in the Diabetes Antibody Standardization Program 2003. The performance of the GADA TR-IFMA and IA-2A ELISA was unsatisfactory, and these tests were not subjected to clinical evaluation.

Conclusions: The GADA ELISA and IA-2A TR-IFMA behave comparably with RBA and are thus suitable for use in the clinical laboratory.

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Type 1 diabetes mellitus (DM1)6 is characterized by autoimmune destruction of β-cells, which leads to insulin deficiency (1). Autoantibodies against β-cells can be detected before clinical onset of DM1 (2, 3) and can help to distinguish type 2 diabetes mellitus (DM2) from latent autoimmune diabetes of the adult (LADA) (4), which may represent one-half of cases of autoimmune diabetes (1). The major autoantibodies in DM1 are against islet cells, insulin, a protein tyrosine phosphatase-like protein (IA-2), and the 65-kDa isoform of glutamic acid decarboxylase (GAD65) (1). Combined analysis of serum GAD65 and IA-2 autoantibodies (GADAs and IA-2As, respectively) may be able to replace the subjective and laborious immunofluorescent detection of islet cell autoantibodies (3, 5, 6) and, unlike insulin-autoantibody testing, help to confirm diabetes classification after insulin replacement therapy has begun.

The standardized radiobinding assay (RBA) detects GADAs and IA-2As in ~70–80% and 40–70%, respectively, of patients with new-onset DM1, whereas the

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5 Nonstandard abbreviations: DM1 and DM2, diabetes mellitus type 1 and 2, respectively; LADA, latent autoimmune diabetes of the adult; IA-2, protein tyrosine phosphatase-like protein; GAD65, 65-kDa isoform of glutamic acid decarboxylase; GADA, GAD65 autoantibody; IA-2A, IA-2 autoantibody; RBA, radiobinding assay; TR-IFMA, time-resolved immunofluorometric assay; NIBSC, National Institute for Biological Standards and Control; and CI, confidence interval.
frequency of detection in the general population is <1% (1, 6). RBAs, although cumbersome and requiring use of $^{35}$S, reportedly provide more precise results and higher diagnostic sensitivity and specificity than ELISAs (2–4).

We compared the analytical and diagnostic accuracy of two commercially available immunoassays, an ELISA and a time-resolved immunofluorometric assay (TR-IFMA), with RBA for measuring GADA and IA-2A concentrations.

Materials and Methods

STUDY PARTICIPANTS

Serum samples obtained between 2000 and 2002 from patients who consecutively attended the Diabetes Clinic of our hospitals were prepared in aliquots and frozen for prospective analysis. The diagnosis of DM1 or DM2 was based on clinical and laboratory assessment according to published recommendations (7). The group of patients classified as new-onset DM1 were newly diagnosed patients whose samples were collected within 14 days of the start of insulin treatment (8). Nonclassified diabetes was diagnosed in patients with an uncertain classification based on atypical onset or disease evolution as judged by clinicians in their initial diagnostic work-up, which included weight, age, ketosis, C-peptide, and insulin dependence. Patients with this diagnosis were selected because one potential clinical use of GADA and IA-2A measurement is to contribute to better diabetes mellitus classification. Samples were classified into four groups as follows, based on the above information: group I, new-onset DM1 (n = 56; 24 women; mean age, 23 years; range, 2–38 years); group II, nonclassified diabetes mellitus (n = 46; 24 women; mean age, 44 years; range, 19–71 years); group III, DM2 (n = 39; 18 women; mean age, 64 years; range, 48–77 years); and group IV, control individuals (n = 74; 44 women; mean age, 47 years; range, 18–83 years), selected from patients without cancer, diabetes mellitus, or cardiovascular or autoimmune diseases, attending for minor surgical procedures.

The Diabetes Antibody Standardization Program distributed in a blind fashion serum sample aliquots from 100 controls and 50 patients with new-onset DM1 to test the sera with any assay currently in use for the detection of diabetes and to determine which samples corresponded to patients with new-onset DM1.

ASSAYS

The assays chosen for this study were the RBA (9), TR-IFMA (DELFIA; Perkin-Elmer; cat. no. 4011-0010 for GADAs and cat. no. 4012-0010 for IA-2As) (2, 3), and ELISA (RSR Ltd.; cat. no. RSR-GDE/96 for GADAs and cat. no. RSR-IAE/96 for IA-2As) (10).

Samples for analytical evaluation were assayed in triplicate in the three assays performed on different days, and accuracy and precision were assessed according to NCCLS recommendations (11). Sera from patients or control individuals used for diagnostic sensitivity and specificity evaluation were analyzed in duplicate. All analyses were performed by two well-trained laboratorians who did not have information regarding previous analytical results or clinical classification. Autoantibody concentration results are reported in WHO international units (kIU/L) and were calculated in relation to the National Institute for Biological Standards and Control (NIBSC) 97/550 reference standard, which was used to calibrate all assays. Diagnostic sensitivity was calculated as the percentage of sera from patients reported as positive based on the laboratory cutoffs for each assay, whereas diagnostic specificity was calculated as the percentage of control sera (group IV) reported as negative based on the same threshold.

Results

Cutoff values were first established to permit special focus on these values in analytical recovery and precision studies. The cutoff values were calculated as the 99th percentiles of autoantibody concentrations of the control population (group IV) after omission of outlying samples: two samples (38.2 and 48.2 kIU/L) for the GADA RBA, one (12.1 kIU/L) for the IA-2A RBA, and none for the remaining of assays, as reported (12). Cutoff values were 24.3 kIU/L [95% confidence interval (95% CI), 22.1–24.8 kIU/L] and 3.9 (2.1–7.2) kIU/L for the GADA RBA and ELISA, respectively, and 9.6 (7.8–9.7) and 25.6 (18.8–31.7) kIU/L for the IA-2A RBA and TR-IFMA, respectively. Cutoff values for the GADA TR-IFMA and IA-2A ELISA could not be established because several assays produced inconsistent results. Thus, cutoff values were assumed to be those recommended by the manufacturers (27.9 kIU/L for the GADA TR-IFMA and 30.0 kIU/L for the IA-2A ELISA).

Analytical Evaluation

We measured recovery of diluted NIBSC 97/550 standard (added concentration) mixed with an in-house negative control (endogenous concentration; see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol50/issue8/). Recoveries at GADA concentrations near the diagnostic cutoff were 88–99% for the RBA, 106% for the ELISA, and 89% for the TR-IFMA. Recoveries at IA-2A concentrations near the cutoff range were 159–162% in the RBA, 48–64% in the TR-IFMA, and 156–221% in the ELISA. Imprecision values (CV) for serial dilutions of the NIBSC 97/550 standard are listed in Table 2 of the online Data Supplement. Within-run imprecision (CVWR) for GADA values near the cutoff was 14% for the RBA, 9% for the ELISA, and 3–10% for the TR-IFMA. The CVWR for IA-2A near the cutoff values was ~14% for the RBA, 9% for the ELISA, and 3–10% for the TR-IFMA. In contrast, the CV for the RBA and ELISA and ≥50% for the TR-IFMA. The detection limits (calculated as the mean signal + 3 SD of a negative serum)
for GADAs were 15.6 kIU/L in the RBA, 3.2 kIU/L in the ELISA, and 22.4 kIU/L in the TR-IFMA, and for IA-2As were 4.0 kIU/L in the RBA, 32.1 kIU/L in the ELISA, and 20.6 kIU/L in the TR-IFMA.

Given our previous experience of poor data replication of plasma samples from controls and patients, we excluded the TR-IFMA GADA and ELISA IA-2A methods from the following clinical evaluation although, in general, we found no major failures during the analytical evaluation and cutoff limit determinations.

**Clinical Evaluation**

The ELISA and RBA GADA showed diagnostic specificities of 99% (95% CI, 93–100%) and 96 (89–99)%, respectively. The highest diagnostic sensitivity for GADA in patients with new-onset DM1 was obtained with the ELISA (89%; 95% CI, 78–95%), whereas the RBA had a sensitivity of 71 (58–82)% in the same group of patients (P <0.03). GADA values in the four groups analyzed are shown in Fig. 1A.

The IA-2A TR-IFMA had a diagnostic specificity similar to that of the RBA [99 (93–110)% and 97 (91–99)% respectively]. The diagnostic sensitivity of the TR-IFMA and RBA in patients with new-onset DM1 was 37 (26–51)% and 50 (37–63)%, respectively (difference not significant). IA-2A TR-IFMA positivity in the group with non-classified diabetes mellitus was not statistically different with respect to RBA (24% and 20%, respectively). No patients clinically classified as having DM2 were positive for IA-2As in either the RBA or TR-IFMA. IA-2A concentrations obtained with the RBA and TR-IFMA in the four groups studied are shown in Fig. 1B.

Results obtained with each assay for patients with new-onset DM1 were subjected to ROC curve analysis (see Fig. 1 in the online Data Supplement). Areas under the ROC curves showed a significant difference (P <0.001) between GADA assays: 0.84 in the RBA (95% CI, 0.77–0.90) and 0.99 in the ELISA (95% CI, 0.95–1.00). IA-2A areas under ROC curves also showed a significant difference (P <0.001) between assays: 0.54 in the RBA (95% CI, 0.45–0.63) and 0.80 in the TR-IFMA (95% CI, 0.72–0.86).

Passing–Bablok regression analysis for GADA yielded the following equation: ELISA = 0.6 RBA + 3.9 kIU/L (95% CI for intercept, −4.5 to −3.0 kIU/L; 95% CI for slope, 0.5–0.7), which shows a high correlation between methods (r = 0.94; P <0.001). A scatter plot of all samples analyzed for GADAs by RBA and ELISA is shown in Fig. 2A. Seventeen of 141 patient samples yielded discordant results: all except one (belonging to the DM2 group) tested positive with the ELISA and negative with the
RBA. Ten of these 17 samples were from patients with new-onset DM1, 4 were from patients with nonclassified diabetes mellitus, and 3 were from the DM2 patient group (potential LADA patients). One control sample was slightly positive with both assays, but two additional samples among the control sera were positive in the RBA.

Regarding IA-2As, Passing–Bablok regression analysis yielded the following equation: TR-IFMA = 2.9 RBA − 1.0 kIU/L (95% CI for intercept, −6.0 to 2.2 kIU/L; 95% CI for slope, 1.9–4.2; \( r = 0.83; P \leq 0.0001 \)). A scatter plot of all samples analyzed for IA-2A is shown in Fig. 2B. Twelve patient samples produced no concordant results: 9 were from patients with new-onset DM1, whereas the others were from patients with nonclassified diabetes mellitus. All but one, which was from a patient with new-onset DM1, tested positive with the RBA and negative with the TR-IFMA. One control sample was positive in the TR-IFMA, whereas two other control samples were positive in the RBA.

The diagnostic sensitivities and specificities obtained in the Diabetes Antibody Standardization Program 2003 for the GADA RBA and ELISA and the IA-2A RBA and TR-IFMA are listed in Table 1.

**Discussion**

The performance of the GADA ELISA was excellent. The recovery, \( CV_{WR} \) and \( CV_T \) near the cutoff value were similar in the RBA and ELISA and were comparable to the intra- and interassay CVs obtained previously (10). The diagnostic accuracies of the RBA and ELISA were also satisfactory, with the latter being slightly better. Because of its slightly better accuracy, and in agreement with other studies (10), the ELISA detected more positive individuals than did the RBA among patients with new-onset DM1, and importantly, this was not accompanied by a lower diagnostic specificity. On the other hand, the ELISA was also slightly more sensitive than the RBA in detecting positivity among patients with DM2, who are likely to have LADA.

The \( CV_{WR} \) for IA-2A concentrations near the cutoff was better for the TR-IFMA than for the RBA, although the \( CV_T \) behaved in an opposite manner at the same threshold. Our imprecision values were similar to those reported previously (3). In Passing–Bablok regression analyses, the IA-2A RBA and TR-IFMA methods showed a proportional difference, which is not important for diagnosis of autoimmune diabetes given that, near the cutoff range, the assays correlated very well. However, this difference could yield a different quantitative risk assessment in the general population or in relatives of patients with DM1 because the IA-2A concentration has been suggested as being related to risk for DM1 (6).

The diagnostic sensitivities of IA-2A measurements obtained with the TR-IFMA and RBA in our patients with new-onset DM1 and in the Diabetes Antibody Standardization Program 2003 for patients with new-onset DM1 were consistent with the previously reported prevalence of IA-2A (40–70%) (1), although lower than the prevalences reported by others using the same methods (3). None of the assays detected positivity in patients with DM2. It is well known that IA-2A prevalence is lower than that of GADAs in patients with LADA (usually <4%) (1). Thus, the number of patients with DM2 studied was too low to yield positive results.

The results obtained in the present study demonstrate that the ELISA for GADA and the TR-IFMA for IA-2A detection represent good alternatives to the corresponding RBAs. The ELISA and TR-IFMA are simple, easy to automate, and constitute highly reproducible methods suitable for routine use in the clinical laboratory. Moreover, both assays are based on reagents that are stable over long periods and avoid the use of harmful radioactive material. One disadvantage of the ELISA and TR-IFMA is the higher sample volume required compared with the RBA, particularly for autoantibody determination in newborns.

Several reagent sets were kindly provided by Perkin-Elmer, Boston, MA (TR-IFMA) and RSR, Cardiff, UK (ELISA). We are grateful to Christine O’Hara for editorial assistance. This study was funded in part by Grants FIS C03-08 and G03/212.

**References**


**Table 1. Diagnostic sensitivity and specificity obtained in the Diabetes Antibody Standardization Program 2003.**

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>Assay</th>
<th>Diagnostic sensitivity, %</th>
<th>Diagnostic specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADA</td>
<td>RBA</td>
<td>84 (72–92)</td>
<td>97 (92–99)</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td>94 (84–98)</td>
<td>98 (93–100)</td>
</tr>
<tr>
<td>IA-2A</td>
<td>RBA</td>
<td>66 (52–78)</td>
<td>100 (96–100)</td>
</tr>
<tr>
<td></td>
<td>TR-IFMA</td>
<td>50 (37–63)</td>
<td>100 (96–100)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All differences between assays for GADAs (RBA vs ELISA) and IA-2As (RBA vs TR-IFMA) were not statistically significant.

<sup>b</sup> Values in parentheses are the 95% CIs.