Protein tyrosine phosphatase-like protein IA2-antibodies plus glutamic acid decarboxylase 65 antibodies (GADA) indicates autoimmunity as frequently as islet cell antibodies assay in children with recently diagnosed diabetes mellitus

HENRIK BORG,¹* PER FERNLUND,² and GÖRAN SUNDKVIST¹

Islet cell antibodies (ICA), the classical autoimmunity marker for insulin-dependent diabetes mellitus (IDDM), are detected in ~85% of children with recently diagnosed diabetes. Because the ICA assay is semiquantitative and difficult to standardize, alternative assays are needed. When glutamic acid decarboxylase 65 (GAD 65) was discovered as a major islet antigen, the measurement of antibodies to GAD 65 (GADA) was considered a good alternative to ICA. Recently, however, we showed that 1 in 3 ICA-positive diabetic patients do not have GADA. Now, antibodies against the protein tyrosine phosphatase-like protein IA2 (IA2-ab) have been detected in IDDM. To find out whether measurements of IA2-ab combined with those of GADA could detect autoimmunity to the same extent as ICA, we have measured all three kinds of antibodies (using radioligand binding assays for IA2-ab and GADA) in 100 recently diagnosed diabetic and 100 control children: ICA were found in 87, IA2-ab in 69, and GADA in 66 of the 100 diabetic patients, whereas in the 100 control children ICA were found in 2, IA2-ab in 1, and GADA in 3. Among the 87 ICA-positive patients, 45 (52%) had both IA2-ab and GADA, 21 (24%) had only IA2-ab, and 16 (18%) had only GADA, whereas 5 (6%) lacked both IA2-ab and GADA. Among the 13 ICA-negative patients, 1 (8%) had both IA2-ab and GADA, 2 (15%) had only IA2-ab, and 4 (31%) had only GADA. Thus, 6 of the 100 patients had neither ICA, IA2-ab, nor GADA. Combining the IA2-ab and GADA assays gave positive results for autoimmunity in 89 of the 100 patients, compared with 87 by the ICA assay. The combination of the IA2-ab and GADA assays appears to be an effective alternative to the ICA assay.

Insulin-dependent diabetes mellitus (IDDM)³ is an autoimmune disease in which destruction of the insulin-producing β-cells leads to a loss of endogenous insulin secretion and thereby a lifelong need for insulin treatment. Islet cell antibodies (ICA, antibodies reacting against the islets of Langerhans) constitute a well-known autoimmune marker of IDDM, which may be detected in 85% of children at the time of diagnosis of the disease [1]. The antigens reactive in the ICA assay were unknown until 1990, when glutamic acid decarboxylase 65 (GAD 65) antibodies (GADA) were discovered as another autoimmune marker of IDDM [2]. GAD 65 was first thought to be the main ICA antigen, but absorption studies with GAD 65 have shown that GAD 65 is not the only antigen in the ICA reactions [3]—in agreement with our recent demonstration that only 2 of 3 ICA-positive sera from diabetic patients contain GADA [4]. The antigen or antigens reacting with the remaining ICA-positive sera (about one-third) therefore remain to be found. Antibodies against a protein tyrosine phosphatase-like protein, IA2, have recently been demonstrated in diabetic patients [5–10]. The aim of this study was to find out whether the assays of GADA and of antibodies against IA2 (IA2-ab) together could detect autoimmunity to the same extent as ICA assays in children with recently diagnosed diabetes.

³ Nonstandard abbreviations: IDDM, insulin-dependent diabetes mellitus; ICA, islet cell antibodies; GAD 65, 65-kDa glutamic acid decarboxylase; GADA, glutamic acid decarboxylase antibodies; IA2, protein tyrosine phosphatase-like protein; IA2-ab, antibodies against IA2; JDF, Juvenile Diabetes Foundation (units).
If so, the combination of IA2-ab and GADA assays could be used instead of the indirect immunofluorescence ICA assay, which is semiquantitative and difficult to standardize.

Materials and Methods

Subjects
Patients' samples were collected consecutively from 100 children with recently diagnosed diabetes mellitus. All were Caucasians, 47 were girls, and their mean ± SD ages were 9 ± 4 years (range 1–15 years). The samples were obtained from 97 of the patients within 2 weeks of diagnosis. An equal number of control samples were collected from 100 subjects randomly selected from 1031 apparently healthy school children [11]. Of these, 49 were girls and their mean ± SD ages were 11 ± 2 years (range 7–13 years).

Reagents
A cDNA (IA2ic) coding for the intracellular domain of IA2 (beginning at amino acid 606), obtained by courtesy of Michael R. Christie (Kings College, London, UK), was provided as an insert in the BamHI restriction site in the pSP64 Poly(A) plasmid vector (Promega) with the coding strand downstream of the SP6 promoter. The construction of the cDNA has been described elsewhere [5]. Competent Escherichia coli DH5 cells were transformed with the vector, and minipreps of the plasmid were done with routine methods [12]. The plasmid DNA obtained was checked by cutting with BamHI restriction enzyme, which gave a fragment of the expected size (1230 bp), and by sequencing with a vector-specific primer (SP6 primer; DNA Technology, Aarhus, Denmark). The sequence obtained was the same as the published cDNA sequence for human tyrosine phosphatase IA2/PTP (Genbank accession L18983) [13] except for a G2072A substitution, a change that should give an Ala to Thr substitution in the expressed protein. TNT Coupled Reticulocyte Lysate System was obtained from Promega, RNAsin from Appliedgene (Illkirch, France), L-[35S]methionine (>1000 kCi/mol) from Amersham, Protein A–Sepharose CL-4B from Pharmacia, and a Multiscreen 96-well filtration system from Millipore. Buffers used in the IA2-ab and GADA assays were "plain" buffer (NaCl 150 mmol/L, Tris 20 mmol/L, pH 7.4, and NaN3 2 g/L), coating buffer (plain buffer plus bovine serum albumin, 10 g/L), and washing buffer (plain buffer plus bovine serum albumin, 1 g/L, and Tween 20, 1.5 mL/L).

Assay Methods
In the IA2-ab assay, 35S-labeled human recombinant IA2 was synthesized by in vitro transcription–translation with a circular pSP64 poly(A) vector with IA2ic insert and TNT Coupled Reticulocyte Lysate System. The yield of the translation product, determined by trichloroacetic acid precipitation, was between 10% and 20% of the total [35S]methionine added. The translation product was not separated from the remaining free [35S]methionine. Overnight incubations with the 35S-labeled IA2 were made at 4 °C in duplicates for each sample. Two aliquots from each incubation were incubated with Protein A–Sepharose on a 96-well filtration plate to collect immunocomplexes. The filter bottom of each well was punched into a scintillation bottle, and the radioactivity was counted (Wallac 1410; Pharmacia). Sera from three blood donors served as negative controls, and plasma from a patient with a high concentration of IA2-ab diluted in negative control serum served as a positive control. The controls were kept as single-use aliquots at −20 °C. The results are presented as an IA2-ab index: 100 × (u − n)/(p − n), where u = CPM of the unknown sample, n = CPM of the negative control, p = CPM of the positive control, and CPM = mean activity (counts/min) of all four measurements for a sample.

The IA2-ab assay has been evaluated in the Juvenile Diabetes Foundation (JDF) First Proficiency evaluation, showing a 100% diagnostic sensitivity and specificity (24 samples evaluated). The imprecision of the IA2-ab assay was monitored by including in each run two control samples, one with a low and one with a high concentration of IA2-ab. The imprecision (total), estimated by analysis of variance of the pooled values for the controls, was 19% at the low concentration and 12% at the high.

GADA were determined by a radioligand assay based on [35S]methionine-labeled human recombinant in vitro transcribed–translated GAD 65 [4]. Evaluation of this assay in the International Diabetes Workshop GADA65 Proficiency Program (No. 2) showed 100% sensitivity and specificity. The imprecision (total) was 17% at the low concentration and 19% at the high.

ICA were determined by a prolonged immunofluorescence assay as previously described in detail [14]. Four pancreata were used in the study. The detection limit (cutoff limit for abnormality) was 2 JDF units for pancreas 1 (used for 75 patients), 4 JDF units for pancreas 2 (used for 21 patients), 6 JDF units for pancreas 3 (used for 4 patients), and 3 JDF units for pancreas 4 (used for the control subjects). In the latest (No. 12) International Diabetes Workshop proficiency test for ICA, our assay showed a sensitivity of 86% and a specificity of 100%. The imprecision (total) of our ICA assay was 34% at 36 JDF units.

Statistics
The nonparametric Mann–Whitney’s test was used to analyze differences in antibody values between patient and control groups, the nonparametric Kruskal–Wallis test to analyze differences in antibody values between patient groups, and the nonparametric Spearman test (rs) to analyze the degree of correlation between the different antibodies. ROC curves and logistic regression analysis were performed with the SAS 6.10/OS/2 Warp Connect software. Data are presented as median ± interquartile range, if not stated otherwise.
Results

The results obtained by the IA2-ab and the GADA assays are shown in Fig. 1. In both assays, there were pronounced differences \((P < 0.0001)\) in the results between the patients and the controls (IA2-ab, \(37.4 \pm 118.7\) vs \(0.3 \pm 0.5\); GADA, \(11.0 \pm 36.2\) vs \(1.2 \pm 2.1\)). The cutoff limits for the IA2-ab (IA2-ab index = 1.0), and GADA assay (GADA index = 4.5) were determined nonparametrically as the 97.5% percentile. Results above the cutoff limits were considered positive. One control subject gave results regarded as outliers, which were excluded; this boy had high IA2-ab (IA2-ab index = 144.1), GADA (GADA index = 40.1), and ICA (192 JDF) concentrations and later developed diabetes.

ICA were found in 87, IA2-ab in 69, and GADA in 66 of the 100 patients. IA2-ab and GADA seemed to be complementary; only half of the ICA-positive patients (45 of 87, 52%) had both IA2-ab and GADA, but as many as 21 of 87 (24%) ICA-positive patients had only IA2-ab, and 16 of 87 (18%) ICA-positive patients had only GADA. Nevertheless, 5 of 87 (6%) ICA-positive patients lacked both IA2-ab and GADA. Among the 13 ICA-negative patients, 1 (8%) had both IA2-ab and GADA, 2 (15%) had only IA2-ab, and 4 (31%) had only GADA. Among the 100 control subjects, 1 had ICA, IA2-ab, and GADA (the “outlier”), 1 had only ICA, none had only IA2-ab, and 2 had only GADA (Table 1). Combining the results of all three assays showed that 94 of the 100 patients had at least one sign of humoral autoimmunity. If only the IA2-ab and GADA assays were used, 89 of the 100 patients were positive for humoral autoimmunity, i.e., a frequency almost the same as with the ICA assay (87 of 100).

Figure 2 shows the ROC curves obtained for the IA2-ab, GADA, and ICA assays, and for a combination of the IA2-ab and GADA assays made by logistic regression analysis, with the sensitivities determined from the results for the diabetic children \((n = 100)\) and the specificities from the control children \((n = 99;\) excluding the outlier). The areas \((\pm SE)\) under the curves for the different assays were \(0.80 \pm 0.03\) (IA2-ab), \(0.87 \pm 0.03\) (GADA), \(0.93 \pm 0.02\) (ICA), and \(0.90 \pm 0.03\) (IA2-ab and GADA combined). Table 2 gives the sensitivities, based on logistic regression analysis, for different combinations of the assays at some clinically relevant specificities. As Fig. 2 and Table 2 demonstrate, the sensitivity of the combination of IA2-ab and GADA assays corresponded well to that of the ICA assay.

Among diabetic children, there were no effects of age (ICA, \(r_s = 0.11\); IA2-ab, \(r_s = 0.05\); GADA, \(r_s = 0.16\) or gender (girls vs boys: ICA, \(96 \pm 198\) JDF vs \(90 \pm 220\) JDF, \(P = 0.71\); IA2-ab index, \(70 \pm 79\) vs \(56 \pm 59\), \(P = 0.31\);
GADA index, $31 \pm 34$ vs $23 \pm 32$, $P = 0.25$) on the antibody values. On the other hand, ICA values correlated with IA2-ab and GADA results, although not too strongly ($r_s = 0.40$ and $0.38$, respectively). Patients who had both IA2-ab and GADA had higher ICA values than those who had only IA2-ab or GADA ($P < 0.05$) or neither ($P = 0.001$). Notably, there was no correlation between IA2-ab and GADA values ($r_s = 0.17$) (Fig. 3). Hence, although both IA2-ab and GADA correlated with ICA, IA2-ab and GADA must reflect different aspects of ICA.

**Discussion**

This study demonstrates that, in newly diagnosed diabetic children, combining the measurements of IA2-ab and GADA detects autoimmunity to the same extent as determination of ICA by indirect immunofluorescence. Therefore, the combination of the assays of IA2-ab and GADA is a good alternative to the ICA assay in the definition of autoimmune diabetes, as also can be inferred from other recent studies [15, 16]. Given the definite technical advantages of the radioligand binding assays in comparison with indirect immunofluorescence assays, the former two assays may be used instead of the ICA assay, at least for screening large populations. Nevertheless, our study also shows that there still is a place for the ICA assay. In fact, 5% of the patients were ICA-positive but negative for both IA2-ab and GADA, and combining ICA with IA2-ab and GADA gave 94% positivity for autoimmunity markers in our newly diagnosed diabetic patients. Hence, ICA evidently reflect antibodies to other antigens.

---

**Table 1. Frequencies of IA2-ab, GADA, and ICA in 100 diabetic and 100 control children.**

<table>
<thead>
<tr>
<th>Antibodies present</th>
<th>Diabetic children</th>
<th>Control children</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA2-ab, GADA, and ICA</td>
<td>45</td>
<td>1$^a$</td>
</tr>
<tr>
<td>IA2-ab and ICA only</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>GADA and ICA only</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>IA2-ab and GADA only</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IA2-ab only</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>GADA only</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>ICA only</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>6</td>
<td>96</td>
</tr>
</tbody>
</table>

$^a$ Boy, 7 years old, who later developed diabetes.

---

**Table 2. Sensitivity for different combinations of assays in 100 diabetic children at clinically relevant specificities.**

<table>
<thead>
<tr>
<th>Specificity, %</th>
<th>IA2-ab</th>
<th>GADA</th>
<th>ICA</th>
<th>IA2-ab, GADA</th>
<th>IA2-ab, ICA</th>
<th>GADA, ICA</th>
<th>IA2-ab, GADA, ICA</th>
</tr>
</thead>
<tbody>
<tr>
<td>99</td>
<td>69</td>
<td>66</td>
<td>87</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>87</td>
</tr>
<tr>
<td>97.5</td>
<td>69</td>
<td>66</td>
<td>87</td>
<td>88</td>
<td>91</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>95</td>
<td>69</td>
<td>72</td>
<td>87</td>
<td>90</td>
<td>91</td>
<td>94</td>
<td>94</td>
</tr>
<tr>
<td>90</td>
<td>71</td>
<td>72</td>
<td>88</td>
<td>91</td>
<td>92</td>
<td>94</td>
<td>95</td>
</tr>
</tbody>
</table>

$^a$ Values for the combination of assays, extracted from ROC curves made by logistic regression analysis (not shown).
as well, possibly including the Glima 38 antigen [17]. The lack of correlation between IA2-ab and GADA observed in this study is in accordance with previous studies [18] that have shown these antibodies to contribute to the ICA immunofluorescence assay independently of each other.

A crucial question is whether IA2-ab, GADA, or both reflect the primary autoimmune process in IDDM. Previous studies have shown that GADA may precede the clinical onset of diabetes by several years [19], which suggests that GAD 65 may be the primary antigen in the putative diabetogenic autoimmune destruction of pancreatic β-cells. Challenging this concept, however, is our observation that, in contrast to ICA, GADA may persist for decades after the onset of clinical diabetes [20] and also may develop after the diagnosis of IDDM [21, 22]. In fact, some studies show ICA but not GADA associated with impairment of β-cell function [21]. In our study, IA2-ab alone occurred in only one control child, a boy who later developed diabetes. Hence, IA2 and not just GAD 65 might be involved in the pathogenic autoimmune process, as recently suggested by others [23]. Therefore, in studies aimed to clarify the pathogenesis of IDDM, determinations of both IA2-ab and GADA seem necessary.

In conclusion, determination of both IA2-ab and GADA by radioligand binding assays based on recombinant antigens gave a frequency (89%) of islet autoimmunity in recently diagnosed diabetic children that corresponded well to that found by the indirect immunofluorescence assay for ICA (87%). Although the combination of IA2-ab, GADA, and ICA assays together gave the highest frequency (94%), at least in the clinical setting, IA2-ab and GADA determinations may be a valuable alternative to the ICA assay.

We thank Michael R. Christie for providing IA2 cDNA; Ingegerd Larsson, Ann Radelius, and Christina Rosborn for valuable technical assistance; and Jan-Åke Nilsson for help with ROC curve constructions. This study was supported by grants from the Child Diabetes Fund, Lundström Foundation, Malmö Diabetes Association, Novo-Nordic Foundation, Research Funds Malmö University Hospital, Swedish Diabetes Association, Swedish Medical Research Council (7507 and 5913), and University Funds Lund University.

References

5. Payton MA, Hawkes CJ, Christie MR. Relationship of the 37,000- and 40,000-Mr tryptic fragments of the islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA2 (ICA512), J Clin Invest 1995;100:6–11.


