Slowly progressive insulin-dependent (type 1) diabetes positive for anti-GAD antibody ELISA test may be strongly associated with a future insulin-dependent state

Yoichi Oikawa¹, Hajime Tanaka¹, Junko Uchida¹, Yoshihiro Atsumi¹, Masaya Osawa¹, Takeshi Katsuki¹, Toshihide Kawai¹ and Akira Shimada²

¹ Department of Internal Medicine, Tokyo Saiseikai Central Hospital, Tokyo 108-0073, Japan
² Department of Endocrinology and Diabetes, School of Medicine, Saitama Medical University, Saitama 350-0495, Japan

Abstract. Slowly progressive insulin-dependent (type 1) diabetes mellitus (SPIDDM), believed to be caused by β-cell destruction through islet-cell autoimmunity, gradually progresses to an insulin-dependent state over time. Although the presence of anti-glutamic acid decarboxylase antibody (GADA) is required for the diagnosis of SPIDDM, a recent change in the GADA assay kit from radioimmunoassay (RIA) to enzyme-linked immunosorbent assay (ELISA) yields mismatched GADA test results between the two kits, leading to confusion in understanding the pathological conditions of SPIDDM in Japan. Thus, this study aimed to clarify the difference in the clinical characteristics of GADA-ELISA–positive and GADA-ELISA–negative patients originally diagnosed as SPIDDM by GADA-RIA test. As a result, 42 of 63 original GADA-RIA–positive SPIDDM patients (66.7%) were found to be GADA-ELISA–positive, whereas the remaining 21 patients (33.3%) were found to be GADA-ELISA–negative. In patients with shorter disease duration, GADA-ELISA–positive patients showed significantly lower serum C-peptide levels than GADA-ELISA–negative patients. Meanwhile, in patients with longer disease duration, serum C-peptide levels were comparably decreased in GADA-ELISA–positive and GADA-ELISA–negative patients. A significant inverse correlation between serum C-peptide level and disease duration was observed in GADA-ELISA–negative patients, but not in GADA-ELISA–positive patients, suggesting that insulin secretory capacity may be gradually impaired over time also in GADA-ELISA–negative SPIDDM patients. In conclusion, physicians should be aware that GADA-ELISA–positive SPIDDM may be strongly associated with a future insulin-dependent state. Meanwhile, physicians should be careful in treating GADA-ELISA–negative SPIDDM patients diagnosed as type 2 DM, and cautiously follow the clinical course, in accordance with SPIDDM.

Key words: Anti-glutamic acid decarboxylase antibody (GADA), Enzyme-linked immunosorbent assay (ELISA), Slowly progressive insulin-dependent (type 1) diabetes mellitus (SPIDDM), Radioimmunoassay (RIA)
Materials and Methods

This study is a cross-sectional observational study. We collected the data of 63 outpatients in Tokyo Saiseikai Central Hospital, who had been previously diagnosed as SPIDDM by GADA-RIA kit according to the diagnostic criteria for SPIDDM (2012) [5]. They were tested for GADA by GADA-ELISA kit between December 2015 and May 2016. The clinical characteristics of all patients [sex, age, body mass index (BMI), disease duration, ad lib plasma glucose level; HbA1c level; serum C-peptide level; therapeutic methods for DM] were obtained.

The serum GADA level at the last measurement by GADA-RIA kit (RiaRSR™ GADAb; RSR Ltd., Cardiff, UK) and that at the first measurement by GADA-ELISA kit (ElisaRSR™ GADAb; RSR Ltd.) were also obtained. The cut-off values of GADA-RIA test and GADA-ELISA test are 1.5 U/mL and 5.0 U/mL, respectively. The lower detection limits for the GADA-RIA and GADA-ELISA tests are 0.11 U/mL and 0.57 U/mL, respectively. The intra- and inter-assay coefficients of variation (CV) for the GADA-RIA test are 3.6–3.7% and 5.5–6.9%, respectively, while those for the GADA-ELISA test were 3.5–8.5% and 5.2–6.4%, respectively (taken from the manufacturer’s data sheets of the two kits; RSR Ltd.). Moreover, the interval between the timing of GADA-RIA test and GADA-ELISA test was recorded. The GADA-RIA and GADA-ELISA tests were outsourced to SRL, Inc., a commercial laboratory in Japan, and performed according to the manufacturer’s instructions. As controls, we collected the data of 38 patients with type 2 DM who had been previously determined to be negative for GADA-RIA test. This study protocol was approved by the Institutional Review Board of Tokyo Saiseikai Central Hospital.

Statistical analysis

Data were presented as the mean ± SD except for GADA, which was presented as the mean ± SE. Group comparisons were performed using Pearson’s Chi-square test, Fisher’s exact test, unpaired t-test and Mann-Whitney U-test as appropriate. Moreover, a correlation between disease duration and serum C-peptide level was analyzed using the Pearson’s correlation coefficient. A p-value of <0.05 was considered significant. Statistical analysis was performed with the SPSS statistics software package.
Results

Clinical characteristics of the patients originally diagnosed as SPIDDM by GADA-RIA test

As shown in Table 1, the mean age and mean disease duration of SPIDDM patients were 59.14 years old and 14.25 years, respectively. Meanwhile, the mean age and mean disease duration of patients with type 2 DM were 61.55 years old and 14.31 years, respectively. No significant difference was found in these two factors between the patients with SPIDDM and type 2 DM patients. There was no significant difference in the population of insulin-treated patients between SPIDDM and type 2 DM patients. Whereas 8 of 38 patients with type 2 DM used insulin secretagogues (i.e., sulfonylurea or glinide), 3 of 63 SPIDDM patients used them at the physicians’ discretion in spite of SPIDDM. The frequency of use of insulin secretagogues was significantly higher in type 2 DM patients compared to SPIDDM patients.

The present study showed that 42 of 63 SPIDDM patients (66.7%) originally diagnosed by GADA-RIA test were later found to be positive for GADA-ELISA test, while 21 of 63 SPIDDM patients (33.3%) were found to be negative by GADA-ELISA. The interval between the last GADA-RIA measurement and the first GADA-ELISA measurement was 2.41 ± 1.84 years.

Next, we divided the original GADA-RIA–positive SPIDDM patients into GADA-ELISA–negative and GADA-ELISA–positive groups, and compared the clinical characteristics between the two groups. As shown in Table 2, the mean DM onset age tended to be lower in GADA-ELISA–positive and GADA-RIA–positive patients than in GADA-ELISA–negative and GADA-RIA–positive patients (43.26 ± 13.36 vs. 48.17 ± 16.00 years old, respectively), though no significant difference between the two groups. The last GADA-RIA value was significantly lower in GADA-ELISA–negative and GADA-RIA–positive patients than in GADA-ELISA–positive and GADA-RIA–positive patients (4.54 ± 0.77 vs. 975.55 ± 558.86, respectively; p <0.01). In GADA-ELISA–negative and GADA-RIA–positive patients, the maximal value of GADA-RIA was 14.0 U/mL and there were only 2 cases with a GADA-RIA value of ≥10 U/mL (11.5 U/mL and 14.0 U/mL).

As shown in Table 2, compared to GADA-ELISA–negative and GADA-RIA–positive patients, the serum C-peptide level was significantly lower in GADA-ELISA–positive and GADA-RIA–positive patients, despite the lower age and shorter disease duration. Accordingly, more GADA-ELISA–positive and GADA-RIA–positive patients were treated with insulin.

Table 1 Clinical characteristics of GADA-RIA–positive SPIDDM patients and patients with type 2 DM

<table>
<thead>
<tr>
<th></th>
<th>SPIDDM (n = 63)</th>
<th>Type 2 DM (n = 38)</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>59.14 ± 13.38</td>
<td>61.55 ± 14.59</td>
<td>NS **</td>
</tr>
<tr>
<td>Sex (Female / Male)</td>
<td>25 / 38</td>
<td>13 / 25</td>
<td>NS *</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>61.64 ± 10.31</td>
<td>68.45 ± 17.69</td>
<td>&lt;0.05 **</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.04 ± 3.50</td>
<td>25.41 ± 5.43</td>
<td>&lt;0.01 **</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>167.87 ± 76.45</td>
<td>151.24 ± 41.04</td>
<td>NS **</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.00 ± 1.88</td>
<td>8.35 ± 1.93</td>
<td>NS **</td>
</tr>
<tr>
<td>Serum C-peptide (ng/mL)</td>
<td>1.01 ± 1.05</td>
<td>2.28 ± 1.55</td>
<td>&lt;0.01 **</td>
</tr>
<tr>
<td>Onset age of DM (years)</td>
<td>45.89 ± 14.36</td>
<td>47.25 ± 13.77</td>
<td>NS **</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>14.25 ± 9.80</td>
<td>14.31 ± 9.79</td>
<td>NS **</td>
</tr>
<tr>
<td>Last GADA-RIA values (U/mL; mean ± SE)</td>
<td>651.88 ± 373.60 (1.5 / 19,000)</td>
<td>– (0.3 / 0.9)</td>
<td>&lt;0.01 #</td>
</tr>
<tr>
<td>Interval between last GADA-RIA and first GADA-ELISA tests (years)</td>
<td>2.41 ± 1.84</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Insulin use (yes / no)</td>
<td>47 / 16</td>
<td>28 / 10</td>
<td>NS *</td>
</tr>
<tr>
<td>Insulin secretagogues use (yes / no)</td>
<td>3 / 60</td>
<td>8 / 30</td>
<td>&lt;0.05 *</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD unless otherwise indicated. * Chi-square test or Fisher’s exact test; ** Unpaired t-test; # Mann–Whitney U-test. ## Insulin secretagogues include sulfonylurea and glinide, but not dipeptidyl peptidase-4 (DPP-4) inhibitor. BMI, body mass index; DM, diabetes mellitus; ELISA, enzyme-linked immunosorbent assay; GADA, anti-glutamic acid decarboxylase antibody; NS, not significant; SPIDDM, slowly progressive insulin-dependent (type 1) diabetes mellitus; RIA, radioimmunoassay.
Lower insulin secretion capacity in GADA-ELISA–positive and GADA-RIA–positive SPIDDM patients with shorter disease duration

As described above, although GADA-ELISA–positive and GADA-RIA–positive patients may show a lower insulin secretion capacity, this finding may be affected by disease duration. Thus, we divided all SPIDDM patients into two groups according to disease duration, shorter (<11 years; n = 32) and longer (≥11 years; n = 31) disease duration, and evaluated the serum C-peptide level; 11 years was the median value of the disease duration of all patients. As a result, in the patients with shorter disease duration, the serum C-peptide level was significantly lower in GADA-ELISA–positive and GADA-RIA–positive patients as compared to GADA-ELISA–negative and GADA-RIA–positive patients (0.70 ± 0.62 ng/mL vs. 2.26 ± 1.60 ng/mL, respectively; p < 0.01) (Fig. 1).

Meanwhile, in the patients with a longer disease duration, serum C-peptide level was decreased and comparable between GADA-ELISA–positive and GADA-RIA–negative and GADA-RIA–positive patients (0.72 ± 1.00 ng/mL vs. 1.24 ± 0.81 ng/mL, respectively) with no significant difference (Fig. 1).

GADA-ELISA–negative and GADA-RIA–positive SPIDDM patients conserved insulin secretory capacity at an early stage, but not at an advanced stage, of the disease process of SPIDDM

Next, we investigated the association between insulin secretory capacity and disease duration. As a result, a significant inverse correlation between serum C-peptide level and disease duration was observed in GADA-ELISA–negative and GADA-RIA–positive patients (Fig. 2A), but not in GADA-ELISA–positive and GADA-RIA–positive patients or type 2 diabetic patients (Fig. 2B and C). Although the patients with type 2 DM were divided into two groups (those...
on insulin and those not on insulin), there was no significant correlation between serum C-peptide level and disease duration, regardless of insulin use status (Fig. 2C). Moreover, in the patients with type 2 DM, serum C-peptide maintained a relatively high level throughout disease duration with or without insulin therapy (Fig. 2C). Concretely, mean serum C-peptide levels were 2.20 ± 0.85 ng/mL in patients with type 2 DM with a disease duration <11 years (n = 15) and 2.34 ± 1.89 ng/mL in those with a disease duration ≥11 years (n = 23) with no significant difference. Similar findings were observed in the patients with type 2 DM with or without insulin secretagogues as well (data not shown). As a result, the time-dependent change in serum C-peptide level apparently differed between the patients with type 2 DM and GADA-ELISA–negative and GADA-RIA–positive SPIDDM (Fig. 2A and C). These findings suggest that the insulin secretory capacity may gradually decrease over time also in the GADA-ELISA–negative patients originally diagnosed as SPIDDM by GADA-RIA test.

Fig. 1 Serum C-peptide levels in GADA-ELISA–positive and GADA-RIA–positive SPIDDM patients and GADA-ELISA–negative and GADA-RIA–positive SPIDDM patients according to disease duration

All SPIDDM patients were divided into two groups with shorter disease duration (<11 years) and longer disease duration (≥11 years), and serum C-peptide levels were compared between GADA-ELISA–negative and GADA-RIA–positive SPIDDM patients (white bar; n = 8 in shorter disease duration and n = 13 in longer disease duration) and GADA-ELISA–positive and GADA-RIA–positive SPIDDM patients (black bar; n = 24 in shorter disease duration and n = 18 in longer disease duration). * p <0.01 by unpaired t-test. NS, not significant.

Fig. 2 Correlation between serum C-peptide level and disease duration

Dot-plots show a correlation between serum C-peptide level and disease duration in GADA-ELISA–negative and GADA-RIA–positive SPIDDM patients (A; n = 21), GADA-ELISA–positive and GADA-RIA–positive SPIDDM patients (B; n = 42) and patients with type 2 DM (C; n = 38). A significant inverse correlation between serum C-peptide level and disease duration was observed in GADA-ELISA–negative and GADA-RIA–positive SPIDDM patients (A; Pearson’s correlation coefficient, denoted as r_p = -0.539, p <0.05), but not in GADA-ELISA–positive and GADA-RIA–positive SPIDDM patients (B) and patients with type 2 DM (C). Moreover, although the patients with type 2 DM were divided into two groups: those who were taking insulin (●; n = 28) and those who were not taking insulin (Δ; n = 10), there was no significant correlation between serum C-peptide level and disease duration, regardless of insulin use status (C). NS, not significant.
Discussion

In general, most SPIDDM patients will need insulin treatment at least more than 3 months after onset (or diagnosis) of DM and frequently progress to an insulin-dependent state [5]. In particular, some previous observational studies of SPIDDM have shown that a DM in the non-insulin-dependent state with GADA-RIA value of ≥10 U/mL is a higher risk factor for progression to insulin-dependent state [7, 8]. According to a recent study [9], 73% of SPIDDM patients with a GADA-RIA value of ≥10 U/mL progressed to the insulin-dependent state for a mean of 107 months. Meanwhile, 27% of SPIDDM patients with a GADA-RIA value of <10 U/mL also progressed to the insulin-dependent state. Therefore, physicians should pay attention to the future risk of progression to insulin-dependent state for all SPIDDM patients, regardless of the GADA-RIA values.

The present study revealed that 33.3% of GADA-RIA–positive SPIDDM patients originally diagnosed by GADA-RIA test were later found to be negative by GADA-ELISA test. As for the mismatch of GADA results between the two tests, our earlier study demonstrated that sera of 25.5% of the SPIDDM patients who were positive for GADA-RIA test were found to be negative for GADA-ELISA test [6]. Moreover, when the GADA-RIA value was limited to <20 U/mL, 40% of GADA-RIA–positive SPIDDM patients were found to be negative for GADA-ELISA test [6]. The rate of mismatch was almost comparable to that observed in the present study (33.3%), and physicians should beware that 30 to 40% of GADA-RIA–positive SPIDDM patients with a GADA-RIA value of <20 U/mL may be found to be negative for GADA-ELISA test [6].

Another possible reason for the GADA test result mismatch lies in the fact that the GADA-RIA test requires a single wash step in the measuring process, possibly leading to a non-specific response and a false–positive result, particularly in the lower GADA range, in contrast to the 3 wash steps in GADA-ELISA test [12, 13]. Furthermore, a lower binding affinity of GADA to GAD65 protein may exist in SPIDDM within a lower GADA range and may not be detected by GADA-ELISA kit, for reasons unknown. Recently, some researchers are seriously concerned that the cut-off value of the GADA-ELISA test (5.0 U/mL) may be high, particularly for the Japanese population, and lead to a false–negative result.

Moreover, the discrepant result between the two GADA tests may be due to differences in the assay
methods, *i.e.*, solid phase (ELISA) versus liquid phase (RIA). Thus far, the sensitivity and specificity of the two tests for discriminating newly diagnosed type 1 DM from normal controls were officially evaluated in the past four international Immunology of Diabetes workshops (Diabetes Autoantibody Standardization Program (DASP) 2010, Islet Autoantibody Standardization Program (IASP) 2012, IASP 2013 and IASP 2015). As a result, the GADA-ELISA test consistently achieved higher sensitivity and specificity levels than the GADA-RIA test (sensitivity/specificity: 74–90%/94–98% for ELISA and 70–86%/92–98% for RIA) in all four programs (taken from the manufacturer’s data sheets of the two kits; RSR Ltd.). Behind this background, the GADA-ELISA assay may rely on the autoantibody forming a bridge between immobilized GAD65 on the plate and biotinylated GAD65 in the solution with detection by streptavidin peroxidase [12, 13]. In fact, it is believed that the bridging conformation favors the recognition of high-affinity antibodies, contributing to the good specificity [14]. Given that researches say that the existence of high-affinity GADA is more closely associated with the progression of type 1 DM [15, 16], the possible high disease activity in GADA-ELISA–positive and GADA-RIA–positive SPIDDM is easily understandable.

The present study demonstrated that, although GADA-ELISA–negative and GADA-RIA–positive SPIDDM patients with a shorter disease duration conserved insulin secretory capacity, those with a longer duration showed a decreased insulin secretory capacity at the same level as GADA-ELISA–positive and GADA-RIA–positive SPIDDM patients with a longer disease duration (Figs. 1 and 2A). Moreover, the time-dependent change in serum C-peptide level apparently differed between the patients with type 2 DM and GADA-ELISA–negative and GADA-RIA–positive SPIDDM (Fig. 2A and C). These findings suggest that a portion of GADA-ELISA–negative and GADA-RIA–positive SPIDDM, all of which showed the GADA-RIA value of <20 U/mL, may progress to the insulin-required state or the insulin-dependent state more slowly as compared to GADA-ELISA–positive and GADA-RIA–positive SPIDDM. This notion is believed to be relatively consistent with the recent study described above, which showed that 27% of GADA-RIA–positive SPIDDM patients with a GADA-RIA value of <10 U/mL progressed to the insulin-dependent state [9]. Thus, even if a patient originally diagnosed as SPIDDM by GADA-RIA test is later found to be negative for GADA-ELISA test, the physician should provide SPIDDM therapy for the patient, but not type 2 DM therapy. Recently, the Japan Diabetes Society also made the recommendation that physicians should provide appropriate therapy for diabetic patients with reference to the GADA-RIA value if the patient had previously taken the GADA-RIA test.

There are several limitations in the present study. First, this is a small sample from a single institution which may raise concerns about generalizing the data, and thus warrants further investigation. Second, due to the nature of the cross-sectional study design, the causal correlation between GADA-RIA or GADA-ELISA values and future progression to the insulin-dependent state remains unknown. To clarify the point, a longitudinal prospective follow-up study will be needed in the future. Third, we could not investigate the relationship between the GADA-ELISA readings and other islet cell-associated autoantibodies (anti-IA-2 antibody, anti-ZnT8 antibody, IAA and ICA) because measuring the islet cell-associated autoantibodies in patients diagnosed as type 1 DM is limited in clinical practice covered by health insurance in Japan. Fourth, the presence of anti-thyroid peroxidase antibody, which was reported to facilitate the estimation of future progression to the insulin-dependent state in SPIDDM regardless of GADA-RIA values [17], could not be obtained in the present study. In the future, we will clarify the association between the values of the anti-thyroid peroxidase antibody test and the GADA-ELISA test. Finally, the continuity or reproducibility of GADA values could not be fully confirmed. Thus, we may have included patients with transient seroconversion of GADA values who should have been considered as having type 2 DM, and not SPIDDM, possibly leading to misinterpretation of data.

In conclusion, the present study demonstrated that GADA-ELISA–positive patients originally diagnosed as SPIDDM by GADA-RIA test showed an attenuated insulin secretory capacity even early in the disease process of SPIDDM. Thus, regardless of previous GADA-RIA values, physicians should beware that GADA-ELISA–positive SPIDDM may progress to the insulin-dependent state in a relatively rapid manner. Moreover, GADA-ELISA–negative patients originally diagnosed as SPIDDM by GADA-RIA
test may also progress to the insulin-required state or the insulin-dependent state, but in a relatively slow manner. Thus, even with negative GADA-ELISA readings, physicians should be careful in treating GADA-ELISA–negative SPIDDM patients diagnosed as type 2 DM, and cautiously follow the clinical course over time, according as SPIDDM.

**Disclosure**

None of the authors has any potential conflict of interest associated with this research.

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**References**