Autoantibody against IA-2 Improves the Test Sensitivity for Insulin-Dependent Diabetes Mellitus in Japanese Patients of Child Onset

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Abstract. We previously reported that IA-2 autoantibodies (Ab) facilitated the diagnosis of Japanese insulin-dependent diabetes mellitus (IDDM), but the number tested was not large enough to investigate whether IA-2Ab can improve the diagnostic accuracy. In this report, sera from 78 patients with less than 2 year-disease duration (the mean (range) ages were 19.2 (6–52) years old) were tested in order to clarify that the combination of IA-2Ab and glutamic acid decarboxylase 65 (GAD65)Ab would improve the test sensitivity for IDDM. Both of the autoantibodies were frequently detected in Japanese abrupt-onset IDDM but the frequency of GAD65Ab was higher than that of IA-2Ab (69% and 47%, respectively, $P=0.024$). The two autoantibodies were discordant in respect to both positivity and titer. The positivity for IA-2Ab decreased with the increasing onset-age of the patients (76, 47, 37, 21% for each quartile of age tested), but the frequency of GAD65Ab was unaffected. Among the youngest quartile (≤ 12 years old), IA-2Ab, in combination with GAD65Ab, significantly improved sensitivity (68% to 92%, $P<0.05$), but when we tested patients over 12 years old, IA-2Ab caused little, if any, improvement in sensitivity. We confirmed that IA-2 antibody was detected in IDDM among Japanese, as seen in Caucasians, but the test sensitivity was improved only in young IDDM patients among Japanese.

Key words: IA-2, GAD65, IDDM, Sensitivity, Radioligand binding assay

(Endocrine Journal 44: 485–491, 1997)

INSULIN-DEPENDENT diabetes mellitus (IDDM) is considered to be an autoimmune disorder in which pancreatic beta cell destruction occurs in a genetically susceptible host [1]. A growing number of autoantibodies reactive with islet antigens have been described, such as islet cell antibodies (ICA) [2], and autoantibody against insulin [3], the 65,000 Mr. isoform of glutamic acid decarboxylase (GAD65Ab) [4, 5], heat shock protein 60 [6], islet

cell antigen 69 [7], carboxypeptidase H [8], and a GM2-1 ganglioside [9].

Recently, antibodies to Mr. 37,000/40,000 tryptic fragments of the islet cell protein were reported to be detected in IDDM [10] and prediabetic twins [11]. In addition, Bingley *et al.* reported that these antibodies improve the prediction of IDDM of islet cell antibody-positive relatives [12]. We previously reported that these antibodies were also detected in Japanese [13], but assay for antibodies to the Mr. 37,000/40,000 tryptic fragments is difficult for general use since the antigens are expressed at low levels in pancreatic islets and insulinoma cells [14].

One of the receptor-type protein tyrosine phosphatases was cloned from pancreatic islets

Received: October 30, 1996

Accepted: March 28, 1997

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cells, named islet cell antigen 512 (ICA512) [15, 16] or IA-2 [17], and reported to be a target antigen for the Mr. 37,000/40,000 tryptic fragments of the islet cell protein [14, 18, 19] and ICA [19, 20]. Our previous report indicated that autoantibody to IA-2 (IA-2Ab) was titrated by a sensitive radioligand binding assay [21] and might be discordant with GAD65Ab [13]. Because of the small number studied, it is not clear whether the combination of the two antibodies is a good screening test for a large population.

In this study, we first compared the frequency and titer of GAD65Ab with those of IA-2Ab in order to confirm the discordance of the two antibodies in IDDM in Japanese patients. Second, we investigated whether age affects the positivity of IA-2Ab, as seen in Caucasians and whether IA-2Ab improves the diagnostic value in combination with GAD65Ab.

Material and Methods

Patients' sera (Table 1)

Sera from 78 IDDM patients, with less than a two year-disease duration, were collected from our hospitals. These patients fulfilled the criteria for IDDM [22] with ketoacidosis at onset. They were further divided into quartiles according to the onset age. Seventy-eight sera from healthy individuals were also included in the study. All subjects gave informed consent for this study, which was approved by the Ethical Committee at Keio University, Tokyo, Japan. All sera were kept frozen at -80°C and all assays were performed on coded samples.

Assay for GAD65 and IA-2

Autoantibodies against GAD65 or IA-2 were detected using the previously described radioligand binding assay [23, 24]. Briefly, a clone of the full-length human islet GAD65 (clone pEx9, kindly provided by Drs. Allan E. Karlsen and Catherine E. Grubin, University of Washington, Seattle, USA, [25]) or the carboxyl part (aa 256–979) of full length human IA-2 [21, 26] (clone ICA512.bdc, kindly provided by Prof. George S. Eisenbarth, the Barbara Davis Center for Childhood Diabetes, University of Colorado Health Center, Denver, USA) was used

Table 1. Characteristics of the study group

	IDDM	Control
Female/Male	47/31	40/38
Mean age ($\pm$ SD) (year old)	19.2 ± 10.0	20.6 ± 2.5
Range (year old)	6–52	16–50
Disease duration (months)	8.8 ± 9.5	

in an *in vitro* transcription and translation reaction to produce ^{35}S -GAD65 or ^3H -IA-2 respectively. The labeled proteins in duplicate were diluted in 48 μl of immunoprecipitation buffer (20 mmol/L Tris, 150 mmol/L NaCl, 0.15% (v/v) Tween 20, 0.1% (w/v) Aprotinin and 10 mmol/L benzamidine, pH 7.4) before the addition of 2 μl of either the serum sample or a positive or a negative standard serum (final serum dilution 1:25). After overnight incubation at 4°C on a rotating platform, the antibody-bound labeled protein was separated from the free antigen by the addition of 50 μl 50% (v/v) protein A-Sepharose. After washes in 200 μl immunoprecipitation buffer, the Sepharose beads were collected into glass vials containing 4 ml scintillation fluid and the immunoprecipitated radioactivity was determined in a liquid scintillation analyzer. With a positive IDDM and negative healthy standard sera, the levels of GAD65Ab or IA-2Ab were expressed as the index: (Unknown sample – Mean of three negative standard sera) / (Positive standard serum – Mean of negative standard sera).

The upper level of normal of the GAD65Ab assay was estimated to be 0.020 [27]. Intra- and inter assay coefficient variations were 3.2% and 4.4% for GAD65Ab (index 0.623). In the First Combinatorial Autoantibody Workshop (Immunology of Diabetes Society, 1995), our GAD65Ab assay showed 74.4% sensitivity and 98.0% specificity. At the workshop, the sensitivity of GAD65Ab in the radioligand assays varied between 54.5 and 76.5% (51 IDDM patients) among 23 laboratories with 95–99% specificity (100–101 healthy controls).

The IA-2Ab index was also normally distributed among healthy Japanese individuals, so that the upper level of normal of the IA-2Ab assay was estimated to be 0.010 (mean + 3SD). The Receiver Operating Characteristic plots also support this cut off level. Intra- and inter assay coefficient variations were 4.5% and 4.2% (index 0.528). With the serum kit for the First Combinatorial

Table 2. Effects of age on the autoantibodies among IDDM

	Control	All	(onset age)			
			≤ 12	13–15	16–25	≥ 26
GAD65Ab only	1 (1%)	26 (33%)	4	6	7	9
IA-2Ab only	1 (1%)	9 (12%)	6	2	1	0
Both	0 (0%)	28 (36%)	13	5	6	4
None	76 (98%)	15 (19%)	2	2	5	6
Total	78	78	25	15	19	19

Autoantibody Workshop, our IA-2Ab assay showed 60.5% sensitivity and 98.0% specificity. At the workshop, the sensitivity of ICA512/IA-2 autoantibodies in the radioligand assays varied between 56.8 and 72.5% among 12 laboratories with 95–99% specificity.

Statistical analyses

The frequency of antibodies was tested by Fisher's exact test or corrected for onset age by the Mantel Haenszel Chi-square test. The degree of correlation between the two antibodies was expressed as the Phi-coefficient. The relationship between the titers of the two autoantibodies was tested by Pearson's correlation coefficient after log transformation. Probability less than 0.05 after correction for the number of tests (GAD65Ab and IA-2Ab) was considered as statistically significant.

Results

Frequency of the autoantibodies in IDDM in Japanese patients

As shown in Table 2, GAD65Ab and IA-2Ab were each detected individually in two different healthy subjects (1/78, 1.3%). GAD65Ab was more frequently detected in 69% (54/78) in Japanese abrupt-onset IDDM than in healthy subjects ($P < 0.00001$). The frequency of IA-2Ab was also higher than that of controls (45%, 35/78, $P < 0.00001$), which was significantly lower than that of GAD65Ab ($P = 0.0017$). Table 2 showed that the two tests were discordant for positivity among all patients tested, since 35 out of 78 patients had only either of the two autoantibodies (Chi-square=0.857, Phi-coefficient=0.133). The titers of the two

Fig. 1. Scattergram for titers of GAD65Ab and IA-2Ab. The titers of the two autoantibodies were not related ($r^2=0.023$, N.S.).

autoantibodies also had no significant correlation (Fig. 1, $r^2=0.023$, N.S.).

Effect of age and disease duration on the frequency of the autoantibodies

The relationship between the age of onset and frequency of antibodies is shown in Fig. 2. GAD65Ab was detected in 68% of the youngest quartile of abrupt-onset IDDM and consistently observed among all quartiles of age. On the other hand, the frequency of IA-2Ab was 76% in the youngest but decreased with the increase in age (47%, 37%, 21%, for each quartile).

Table 3 showed the disease duration and positivity of the autoantibodies. The frequency of IA-2Ab among the youngest quartile (equal to or less than 12 years old) with disease duration less than one year did not differ from patients with the disease for more than one year (78.6%, 11/14 and

72.7%, 8/11, respectively). This was also observed in GAD65Ab (71.4%, 10/14 and 63.6%, 7/11). The Mantel Haenszel Chi-square test verified that both antibodies were detected with an insignificant difference between less than one-year and more than one-year of disease duration after correction for onset-age.

Adding the test results for IA-2Ab to those for

GAD65Ab tended to improve the sensitivity among all patients (69%, 54/78 to 81%, 63/78). This improvement reached statistical significance in the youngest quartiles (68%, 17/25 to 92%, 23/25, $P=0.020$), but when we tested the patients over 12 years old, IA-2Ab added little, if any, improvement in sensitivity.

Discussion

Antibodies to Mr. 37,000/40,000 tryptic fragments of the islet cell protein and their target antigen, ICA512 [15, 16], IA-2 [17, 18], or IA-2 beta [28], were reported to improve the ability to predict IDDM [11, 29]. Because it was also reported that IA-2Ab and GAD65Ab can be measured with a single assay [19, 30], GAD65Ab, in combination with IA-2Ab, would replace ICA and be a good screening test for a large population [31].

In this study, we clarified that IA-2Ab was detected with a sensitive radioligand binding assay in 76% of young IDDM in Japanese patients (≤ 12 years old), comparable to the frequency reported in Caucasians (51% of patients under the age of 20 years [20], 59% of patients under the age of 15 years [19]). Our result showing that IA-2Ab was frequently detected among the patients with a disease duration of more than one year, differs from a previous report [20]. That report indicated that the frequency of IA-2Ab detected by ELISA was not significantly higher even among the younger IDDM patients with a disease duration of more than 1 month, in comparison with the healthy control subjects. Our report determined that IA-

Fig. 2. Frequencies of GAD65Ab and IA-2Ab according to patient's age. Shaded and unshaded bars indicate IA-2Ab and GAD65Ab, respectively. Numbers indicate quartiles of onset age. 1: ≤ 12 years old, $n=25$, 2: 13–15 years old, $n=15$, 3: 16–25 years old, $n=19$, 4: ≥ 26 years old, $n=19$. GAD65Ab was detected in 68% of the youngest quartile of abrupt-onset IDDM and consistently observed among all quartiles of age. On the other hand, the frequency of IA-2Ab was 76% in the youngest but decreased with the increase in age (47%, 37%, 21%, for each quartile).

Table 3. Duration and positivity for the autoantibodies among IDDM

	≤ 12	13–15	16–25	≥ 26 (onset age)
GAD65Ab				
Disease duration				
Onset	0% (0/1)	67% (2/3)	64% (9/14)	67% (6/9)
0–12 Months	71% (10/14)	89% (7/8)	69% (11/16)	60% (9/15)
13–24 Months	64% (7/11)	57% (4/7)	66% (2/3)	100% (4/4)
IA-2Ab				
Disease duration				
Onset	100% (1/1)	33% (1/3)	21% (3/14)	22% (2/9)
0–12 Months	79% (11/14)	38% (3/8)	25% (4/16)	20% (3/15)
13–24 Months	73% (8/11)	57% (4/7)	100% (3/3)	25% (1/4)

2Ab, as well as GAD65Ab, was similarly detected in patients with a disease duration of less and in those with a disease duration of more than 1 year. The discrepancy between the results of the two reports should be attributed to the higher sensitivity of the radioligand binding assay compared to ELISA [21]. For example, the lower IA-2Ab titer, which was not detected by ELISA, may have existed in sera from the long disease duration IDDM in the previous report.

We next confirmed the previous observation that GAD65Ab and IA-2Ab were discordant with respect to both the frequency and titer of the autoantibodies [11, 19-21, 29], which was also suggested in our previous report [13]. We also observed that the frequency of IA-2Ab constantly decreased with the increase in the patients' age, whereas GAD65Ab was detected at the same frequency regardless of age. This was so even at the onset of the disease (≤ 1 month). This relationship also was observed in Caucasians by Bonifacio *et al.* who reported that IA-2Ab was detected in 59% of patients under the age of 15 years, while only 39% were over 15 years [19].

Thus, in this study, IA-2Ab in combination with GAD65 Ab significantly improved sensitivity in the youngest quartile (≤ 12 years) of age tested. In contrast, only one of the 12 GAD65Ab-negative patients (≥ 16 years), was positive for IA-2Ab, so that IA-2Ab added little, if any, improvement as to sensitivity.

In Japanese, approximately 20% of IDDM patients have a non-insulin-dependent stage and this type of IDDM has been referred to as slowly progressive IDDM [32]. Therefore, Japanese NIDDM patients are considered to be at risk of

developing IDDM and are an adequate population for screening. Our previous report suggests that the test for GAD65Ab is a suitable marker for insulin-dependency in Japanese NIDDM patients [24]. Since patients with slowly progressive IDDM are frequently seen over the age of 15 years, the test for IA-2Ab did not improve sensitivity as seen in this study [33]. We shall further investigate whether the test for IA-2Ab in combination with that for GAD65Ab will improve the positive predictive value for insulin-dependency among Japanese NIDDM.

In conclusion, IA-2Ab autoantibody was frequently detected by the radioligand binding assay in IDDM in Japanese patients as well as Caucasians. We confirmed previous observations indicating that IA-2Ab is discordant with GAD65Ab and is more frequently detected in younger patients. The test for IA-2Ab in combination with that for GAD65Ab improves the test sensitivity for IDDM patients aged under 15 years old.

Acknowledgments

We specially thank Prof. Åke Lernmark (Department of Medicine, University of Washington, Seattle, USA) for his critical review of the manuscript. We are also grateful to Drs. Allan E. Karlson and Catherine E. Grubin (University of Washington, Seattle, USA) and Prof. George S. Eisenbarth (the Barbara Davis Center for Childhood Diabetes, University of Colorado Health Center, Denver, USA) for providing human GAD65 and ICA512.bdc clones.

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