Antibodies to the 37,000-Mr Tryptic Fragment of Islet Antigen were Detected in Japanese Insulin-Dependent Diabetes Mellitus Patients

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Abstract. Sera from 30 Japanese insulin-dependent diabetes mellitus (IDDM) patients of short duration were examined to determine whether they had antibodies to proteolytic fragments of islet antigen, the molecular weights of which were 37,000 and/or 40,000 Mr (37KAb). The median age and disease duration of the patients were 13 (range; 6–22) years old and 12 (range; 0–24) months, respectively. Twelve out of the 30 IDDM patients (40%) had 37KAb, while none of the 16 control subjects was positive for 37KAb. The frequency of the 37KAb was not correlated with disease duration tested. We further investigated the antibodies to ICA512, by radioligand binding assay, which has been proposed to be a target antigen for the 37KAb. Twenty-two (73.3%) patients had antibodies to ICA512 (ICA512AA), but none of the control subjects did. The levels of ICA512AA, which were described as indexes using standard sera, were significantly higher in the patients than in the control subjects (1.436 ± 2.674 and 0.001 ± 0.002 , respectively, $P < 0.05$). The frequency of antibodies to glutamic acid decarboxylase 65 (GAD65Ab) was also higher in the patients than in the control subjects (70% and 0%, respectively), but 7 out of 9 GAD65Ab-negative patients had ICA512AA and/or 37KAb. Since 93% of the IDDM patients had at least one of these antibodies, combined analysis with 37KAb, ICA512AA, and GAD65Ab facilitates diagnosis of Japanese IDDM.

Key words: Insulin-dependent diabetes mellitus, Anti-37KDa antibodies, ICA512, Glutamic acid decarboxylase, Diagnosis

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INSULIN-dependent diabetes mellitus (IDDM) is acknowledged to be an autoimmune disease [1]. Several autoantibodies, such as islet cell antibodies (ICA) [2, 3], insulin autoantibodies (IAA) [4], and antibodies to islet protein of 64,000-Mr (64KD antigen) [5] have been reported to be detected in sera of the IDDM patients and their relatives. Re-

cently, glutamic acid decarboxylase has been identified as the 64KD antigen [6]. Since the antibodies to glutamic acid decarboxylase 65 (GAD65Ab) are frequently detected in Japanese IDDM patients [7, 8] as well as in Caucasian patients, the detection of GAD65Ab is considered to be useful for the diagnosis and the prediction of IDDM. But it has been reported that some ICA-positive patients with autoimmune disease other than IDDM had a high titer of GAD65Ab [9], and that approximately 20% of nondiabetic identical twins with GAD65Ab did not become IDDM patients [10].

By studying antibody binding to proteolytic frag-

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ments of islet antigen, Christie *et al.* [11] identified two distinct antibodies to the 64KD antigen in sera from IDDM patients—antibodies to 50,000 Mr fragments (50KAb) and to 37,000/40,000-Mr fragments (37KAb). The 50,000-Mr fragments were reported to be derived from GAD65 whereas 37KAb was considered to recognize a protein distinct from GAD65, since they did not precipitate GAD65 [12]. Previous reports have shown that about 70% of newly diagnosed IDDM patients had 37KAb [11], and that a 100% positive predictive value was found for 37KAb, in association with ICA, in first-degree relatives of IDDM patients [13].

In this study, we investigated whether or not Japanese IDDM patients had 37KAb. We also examined antibodies to an islet cell antigen 512 (ICA512), which is a novel islet antigen relating to 37KAb [14, 15]. In addition, we compared the frequency of 37KAb and antibodies to ICA512 to that of GAD65Ab.

Materials and Methods

Patients

The subjects of the current study consisted of 30 Japanese IDDM patients with short duration (within 2 years from diagnosis). IDDM was diagnosed according to standard National Diabetes Data Group criteria [16]. All patients started insulin at the time of diagnosis and remained insulin-dependent thereafter. Patients had a median age of 13 years (range 6 to 22 years), and their median disease duration was 12 months (range 0 to 24 months) (Table 1).

Control subjects

Samples were collected from 16 healthy control subjects who had a median age of 26 years (21 to 33 years). All subjects gave informed consent for this study. All sera were kept frozen at -80°C and all assays were performed on coded samples.

Antibody analysis

37KAb: The method originally described by Christie *et al.* was used [11]. Rat insulinoma cell line (RINm5F) [17] maintained in RPMI 1640 medium containing 10% fetal bovine serum was

Table 1. Characterization of IDDM patients and controls

	IDDM	Control
Number	30	16
Age (range)	13 (6–22)	26 (21–33)
Sex (M/F)	14/16	9/7
Disease duration (range)	12 (0–24)	

Age (year) and disease duration (month) were described as median (range).

kindly provided by Dr. Kiyohiko Negishi (Saitama Medical College, Saitama, Japan). Subconfluent RINm5F cells in a 162 cm² tissue culture flask, which contained about 1×10^8 cells, were radiolabeled in methionine-free RPMI 1640 medium with 1 mCi of ³⁵S-methionine (Amersham, Buckinghamshire, England) for 5 h at 37 °C. Radiolabeled insulinoma cells were then homogenated on ice by 20 passes of a motor driven homogenizer in 1 ml of 0.25 M sucrose, 10 mM Hepes (pH 7.4), 0.5 mM L-methionine, 10 mM benzamidine, 0.1 mM *p*-chloro-mercuriphenylsulphonic acid and 0.5% (wt/vol) aprotinin. The homogenate was centrifuged at 10,000 g for 30 min at 4 °C. The particle fraction was resuspended in 200 μl of 10 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 mM methionine, 10 mM benzamidine and 0.5% aprotinin, and was centrifuged at 10,000 g for 15 min. The pellet was resuspended in 400 μl of 10 mM Hepes (pH 7.4) and 150 mM NaCl, and centrifuged at 10,000 g for 15 min. The pellet was then resuspended in 10 mM Hepes (pH 7.4) and 150 mM NaCl, and 0.5 mg/ml of trypsin (Sigma, St Louis, Mo, USA) was added to the suspension followed by incubation for 15 min on ice. Digestion was stopped by the addition of an equal volume of 10 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM benzamidine, 0.1 mM *p*-chloromercuriphenylsulphonic acid, and 0.5 % (wt/vol) aprotinin, and the suspension was centrifuged at 10,000 g for 15 min at 4 °C to remove particulate material. The supernatant was initially precleared by incubation with 50 μl of normal human sera for 2 h at 4 °C followed by binding to 100 μl of 50% (v/v) protein A-Sepharose (Zymed, San Francisco, CA, USA) for 45 min at 4 °C. A second preclearance was performed with 50 μl of normal human sera for 18 h at 4 °C and 100 μl of 50% (v/v) protein A-Sepharose for 45 min. The extract containing 1,250,000 cpm of protein was incubated with 12.5 μl of serum tested for 5 h at 4 °C and the

immune complexes were isolated on 50 μ l of 50% (v/v) protein A-Sepharose. Immunoprecipitates were washed five times before denaturation. Elution and electrophoresis on 10% SDS-polyacrylamide gels were performed, and radioactive immunoprecipitates were visualized by autoradiography.

Assay for GAD65 and ICA512: Autoantibodies to GAD65 or ICA512 were detected by the previously described radioligand binding assay [18, 19]. Briefly, a clone of the full-length human islet GAD65 (pEx9, kindly provided by Drs. Allan E. Karlsen and Catherine E. Grubin, University of Washington, Seattle, USA [20],) and the carboxyl part of full length IA-2 (ICA512.bdc, kindly provided by Prof. George S. Eisenbarth, the Barbara Davis Center for Childhood Diabetes, University of Colorado Health Center, Denver, USA) were used in an *in vitro* transcription and translation reaction to produce 35 S-GAD65 and 3 H-ICA512.bdc, respectively. The labeled proteins in duplicate were diluted in 48 μ l 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 μ l 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 adding 50 μ l 50% (v/v) protein A-Sepharose. After washes in 200 μ l 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.

Using a positive IDDM and negative healthy standard sera, the levels of GAD65Ab or autoantibodies to ICA512AA (ICA512AA) 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 or ICA512AA assay was estimated to be 0.020 [18] or 0.010 (mean + 3 SD of the healthy controls), respectively. Intra- and inter-assay coefficient variations were 3.2% and 4.4%, 4.5% and 4.2%, for GAD65Ab and ICA512AA, respectively. In the First GADA Proficiency Program (Immunology of Diabetes Society, 1995), our GAD65Ab assay showed 100% sensitivity and 100% specificity.

Statistical analysis

Statistical analysis was performed by Student's *t*-test and Fisher's exact test, with a Stat-View Macintosh program.

Results

37KAb

Twelve out of 30 IDDM patients (40%) were positive for 37KAb, while none of the control subjects had 37KAb (Fig. 1, Table 2). When we classified IDDM patients into two groups by disease duration (less than or equal to 12 months, median duration, and greater than 12 months), the frequencies of 37KAb were similar in both the groups tested (Table 3).

ICA512AA

Twenty-two out of 30 IDDM patients (73.3%) were positive for ICA512AA, but none of the controls had ICA512AA (Table 2). The level of ICA512AA in IDDM patients was significantly higher than that in control subjects (1.436 ± 2.674 and 0.001 ± 0.002 , respectively, $P < 0.05$). The fre-

Fig. 1. Immunoprecipitation of the 37,000/40,000-Mr islet antigen from insulinoma cell (RIN5mF) extracts with sera of IDDM patients (lanes 2 and 3) and healthy control subjects (lanes 4 and 5). Lane 3 was considered to be positive. Upper and under arrows indicated 40,000 and 37,000-Mr, respectively. Lane 1 was a standard molecular weight marker, the band of which showed 46,000 Mr.

Table 2. Frequency of the 37KAb, ICA512AA, and GAD65A6

	IDDM	Control
37KAb	40% (21/30)	0% (0/16)
ICA512AA	73.3% (22/30)	0% (0/16)
GAD65Ab	70% (21/30)	0% (0/16)

Table 3. The relation between disease duration and frequencies of antibodies

Duration (mo)	≤ 12	12 >
37KAb	41.2% (7/17)	38.5% (5/13)
ICA512AA	64.7% (11/17)	84.6% (11/13)
GAD65Ab	76.4% (13/17)	61.5% (8/13)

quency of ICA512AA in IDDM patients was not correlated with disease duration tested (Table 3).

GAD65Ab

Twenty-one out of 30 IDDM patients (70%) were positive for GAD65Ab, whereas all of the control subjects were negative for GAD65Ab (Table 2). The level of GAD65Ab in IDDM patients was significantly higher than in control subjects (0.651 ± 1.277 and -0.002 ± 0.006 , respectively, $P < 0.05$). The frequency of GAD65Ab in IDDM patients was not correlated with disease duration (Table 3).

37KAb, ICA512AA in combination with GAD65Ab

Although the frequency of GAD65Ab in IDDM patients was high (70%), seven out of 9 GAD65Ab-negative patients were positive for 37KAb and/or ICA512AA (Fig. 2). In other words, 28 out of 30 IDDM patients (93.3%) had at least one of the three antibodies.

Discussion

IDDM is the result of the specific destruction of pancreatic beta cells by an immune-mediated mechanism [1]. Several autoantibodies, such as ICA [2, 3], IAA [4], and GAD65Ab [6], have been reported to appear at prediabetic and diabetic stages of the disease. Since the assay for the GAD65Ab (radio-ligand binding assay) is easier to perform and the sensitivity of the assay is high (76.2%) [21], the

Fig. 2. Antibody combinations showing the number of individuals within each category.

detection of GAD65Ab is considered to be useful for the diagnosis or the prediction of IDDM.

On the other hand, Christie *et al.* have identified antibodies to islet 64KD antigen in sera from IDDM patients, which was distinct from GAD65 and cleaved by trypsin to 37,000- and 40,000-Mr proteolytic fragments [11]. Antibodies to these antigens (37KAb) are frequently detected in sera from recent onset IDDM patients and are very closely associated with diabetes development in identical twins [10], and first-degree relatives of IDDM patients [13] and ICA-positive schoolchildren [22]. To identify whether 37KAb is associated with Japanese IDDM, we examined 30 recent onset Japanese IDDM patients in this study. Twelve out of 30 patients (40%) were positive for 37KAb, but none of the control subjects had 37KAb. Two out of the 37KAb-positive patients were negative for GAD65Ab. Although the frequency was lower than those previously reported, we were able to clarify that Japanese IDDM patients also had 37KAb, which may improve the sensitivity of diagnosis of IDDM. It was reported that the proportion of 37KAb-positive sera decreased with age, i.e., 94% of younger patients (0–4 years old) were positive for 37KAb, whereas only 67% of older patients (10–14 years old) had 37KAb [23]. In addition, Bingley *et al.* [13] reported that 37KAb is associated with a high titer of ICA, the level of which is known to decrease rapidly after the onset of overt diabetes. In our study, the median age and disease duration of the patients were 13 years old (range; 6–22) and 12 months (range; 0–24), respectively. The higher age and longer disease

duration than those in previous reports may have decreased the frequency of 37KAb in our study.

One of the target antigens for the 37KAb was recently cloned from pancreatic islet cells and reported to contain a domain homologous to the receptor-linked family of protein tyrosine phosphatase [14, 15]. The antigen was designated as ICA512 [24, 25] or IA2 [14, 26] (the DNA sequence of IA2 is nearly identical to ICA512 but has 310 additional bases at 5' end [15]). Since the carboxyl part of full length IA-2 is considered to contain main epitopes recognized by IDDM patients [15], we examined antibodies to ICA512.bdc (ICA512AA) which has the carboxyl part of IA-2 in this study. Our study demonstrated the high frequency of ICA512AA in IDDM patients (73.3%), and the level of ICA512AA in these patients was significantly higher than that of control subjects. Although ICA512 was proposed to be a target antigen for 37KAb, 12 out of 22 ICA512AA-positive patients were negative for 37KAb. One of the reasons for this discrepancy may be the different methods used; it may be difficult to detect a low titer of 37KAb because the 37,000-Mr islet antigen was expressed at low levels in pancreatic or insulinoma cells [15]. On the other hand, the radioligand assay used for ICA512AA showed high sensitivity, as observed for GAD65Ab. We can say that the detection of ICA512AA by radioligand assay improved the analysis of 37KAb, but we should further investigate whether there are new candidate antigens for 37KAb other than ICA512, because there were two 37KAb-positive patients who were negative for ICA512AA.

In this study, the frequency of ICA512AA in patients with shorter disease duration (≤ 12 months) was similar to that in those with longer disease duration (> 12 months, ≤ 24 months). Although we did not test patients whose disease duration were longer than 24 months, ICA512AA may persist for

at least 2 years in Japanese IDDM patients. This result was different from previous report, in which the frequency of ICA512AA decreased within one month from the development of IDDM [27].

The frequency of GAD65Ab was also higher in the patients than in the control subjects in this study, but 7 out of 9 GAD65Ab-negative patients had ICA512AA and/or 37KAb. This difference means that ICA512AA and/or 37KAb facilitated the diagnosis of IDDM. In fact, 93% of IDDM patients had antibodies to at least one islet antigen in the present study. Although ICA have been considered as a golden standard for immunological markers in predicting IDDM, it is possible to say that a combination of these antibodies, GAD65Ab and 37KAb or ICA512AA, supplant the ICA assay.

In conclusion, we demonstrated that Japanese IDDM patients frequently had 37KAb and ICA512AA, as well as GAD65Ab. This combination analysis facilitated the diagnosis of IDDM. We have to further investigate the predictive value of this combination analysis in first-degree relatives of IDDM patients, newly diagnosed diabetic patients without insulin therapy, and in the general population.

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