Autoantibodies to 64,000-M_r islet cell protein in long-term Type 1 (insulin-dependent) diabetic patients

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Summary. Autoantibodies to the 64,000-M_r (64K) islet cell protein, identified as glutamic acid decarboxylase, were assayed in 46 Type 1 (insulin-dependent) diabetic patients with a disease duration of more than 5 years. Of 46 Type 1 diabetic patients, 18 (39.1 %) were found to be positive for 64K antibodies and 12 of these patients had been diagnosed with autoimmune thyroid disease. Serum C-peptide levels were not detectable in 15 of 18 patients positive for 64K antibodies. The samples were also tested for titres of islet cell antibodies. Islet cell antibodies were detected in 15 (32.6 %) of the 46 patients and all the islet cell antibody positive patients were also found to be positive for 64K antibodies. Fur-

thermore, of these 15 patients 12 had previously been diagnosed with autoimmune thyroid disease. A correlation between levels of 64K antibodies and islet cell antibody titre revealed that higher levels of 64K antibodies were observed in patients who had higher islet cell antibody titre. These results demonstrate that most long-term Type 1 diabetic patients with 64K antibodies were also positive for islet cell antibodies complicated by autoimmune thyroid disease.

Key words: Type 1 (insulin-dependent) diabetes mellitus, 64,000-M_r islet protein autoantibodies, islet cell antibodies, autoimmune thyroid disease, long-term diabetes.

The clinical onset of Type 1 (insulin-dependent) diabetes mellitus is associated with a specific loss of the pancreatic Beta cells and an autoimmunity to the pancreatic Beta cells which may play a role in the pathogenic process. Since the presence of islet cell antibodies (ICA) in Type 1 diabetes was reported [1], many factors concerning its aetiology have been investigated using immunogenetic techniques [2–4]. ICA can be detected in almost all Type 1 diabetic patients within several months after onset of the disease, but rapidly disappear during the course of the disease. However, ICA persist in some Type 1 diabetic patients with concomitant autoimmune endocrine disorders [4–6]. On the other hand, several studies have reported that autoantibodies to the 64,000-M_r islet cell protein (64K antibodies) are detected in 70–90 % of newly-diagnosed Type 1 diabetic patients [7–11]. Recently, it was demonstrated that the 64K antigen is glutamic acid decarboxylase (GAD), which is present in the islets as well as in the brain [12]. The 64K antibodies have been shown to appear several years before the onset of disease and in some cases before the detection of ICA [8], and tend to persist compared to ICA despite undetectable serum C-peptide levels [10]. The correlation between 64K antibodies and ICA is unclear however, and there have been no reports regarding the determination of 64K antibodies in Type 1

diabetic patients with long-term diabetes. In the present study, 64K antibodies were assayed in Type 1 diabetic patients whose disease duration was more than 5 years in order to investigate the characteristics of the 64K-antibody-positive Type 1 diabetic patients with long-term diabetes.

Subjects and methods

Subjects

Sera were obtained from 46 patients with Type 1 diabetes with disease duration of more than 5 years. All of the patients were seen in our department at the Nagasaki University Hospital during the period from 1985 to 1991 and were diagnosed according to the National Diabetes Data Group report. The diagnosis of Type 1 diabetes was based on the occurrence of ketoacidosis at onset or a history of being ketosis-prone, daily insulin requirements of $0.7 \text{ IU} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, postprandial C-peptide excretion and serum C-peptide responses to glucagon (1 mg i.v.). The age at examination, age at onset of the disease and duration of the disease in 46 patients (14 males and 32 females) were 34.3 ± 14.3 (mean $\pm$ SD) years, 21.9 ± 12.7 years and 12.4 ± 7.2 years, respectively. Details of the study group are given in Table 1. The diagnosis of autoimmune thyroid disease (AITD) was based on clinical symptoms of thyroid dysfunction, presence of goitre, levels of serum thyroid hormones, thyroid stimulating hormone (TSH) and elevated anti-thyroidal autoantibodies. ¹²⁵I thyroi-

Table 1. Description of study group characteristics of 46 Type 1 (insulin-dependent) diabetic patients whose duration of the disease is more than 5 years

	Number of subjects	Sex (male/female)
Total subjects	46	14/32
Age at sampling (years)	34.3 ± 14.3	
Age at onset (years)	21.9 ± 12.7	
Duration of diabetes (years)	12.4 ± 7.2	
ATMA positive	22 (47.8%)	6/16
ATGA positive	8 (17.4%)	1/7
AAA positive	0 (0%)	0/0
AGA positive	0 (0%)	0/0
AITD	20 (43.5%)	6/14
Postprandial CPR (< 0.3 ng/ml)	38 (82.6%)	12/26

Values are given as mean ± SD except where indicated. Values in parentheses are percentages. ATMA, anti-thyroidal microsomal antibodies; ATGA, anti-thyroglobulin antibodies; AAA, anti-adrenal antibodies; AGA, anti-gastric antibodies; AITD, autoimmune thyroid disease; CPR, C-peptide immunoreactivity

dal uptakes and thyroidal fine-needle aspiration biopsy were also performed.

Islet cell function

Postprandial serum C-peptide and serum C-peptide response to glucagon were measured to evaluate insulin secretion. Glucagon tests were performed after an overnight fast. Blood samples were drawn at 5 min before and at 3, 5, 10, 15 and 30 min after the i. v. injection of 1 mg glucagon (Novo Co., Ltd., Copenhagen, Denmark). C-peptide was measured by radioimmunoassay (RIA) using commercial kits (C-peptide RIA; Daiichi Ri Co., Ltd., Tokyo, Japan). The assay range of C-peptide is 0.3–30 ng/ml. One of the criteria for insulin-dependency is a serum C-peptide level of 1.8 ng/ml at 3 min after the glucagon injection.

Assay of ICA

ICA were detected by an immunoenzymatic method using fresh frozen sections of human pancreatic tissues and peroxidase-labelled protein A on serial dilutions of serum samples as previously described [6, 13, 14]. Using this method, our laboratory participated in the ICA Proficiency Test under the auspices of the Immunology and Diabetes Workshop and the evaluation of our results in the fourth ICA Proficiency Test were as follows: lab validity, consistency, sensitivity and specificity were 97%, 95%, 94% and 100%, respectively. To facilitate interlaboratory comparison, titres have been converted to Juvenile Diabetes Foundation units by comparison with a standard curve constructed with a standard reference serum. We also examined the islet cell reactivity of ICA-positive sera by double staining with anti-glucagon antibodies. Two-colour double antibody immunofluorescence labelling was carried out on fresh frozen sections of human pancreatic tissues. Fluorescein isothiocyanate-conjugate sheep anti-human IgG (1:100 dilution; Cappel, Malveru, Pa., USA) was used to visualize ICA binding and rhodamine-labelled goat anti-rabbit IgG (1:50 dilution; Protos Immuno Research, San Francisco, Calif., USA) was used to detect anti-glucagon antibodies (1:100; Linco Research, Inc., St. Louis, Mo., USA).

Immunoprecipitation of 64,000- M_r islet-cell proteins

Islets from 250–350 g male Wistar rats were isolated by collagenase digestion of the pancreas. Islets were cultured for 1 day in RPMI 1640 medium supplemented with 0.35 g/l NaHCO₃, 10 mmol/l Hepes (pH 7.4), 10% (volume/volume, v/v) fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Islets were individ-

ually selected under a stereomicroscope and transferred to fresh medium supplemented with 1% (v/v) normal human serum. Cultured islets were washed twice by centrifugation (2 min, 200 g) in methionine-free RPMI 1640 medium supplemented with 18 mmol/l glucose, 2% (v/v) normal human serum, penicillin and streptomycin. Islets (2000 islets/ml) were first incubated in this medium for 60 min before [³⁵S] methionine (1.0 mCi/ml, Amersham International, Amersham, Bucks, UK; > 500 Ci/mmol) was added. After incubation for 4 h at 37°C, the labelled islets were washed twice in RPMI 1640 medium containing 0.5 mmol/l methionine and once in buffer containing 20 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 0.5 mmol/l methionine and 10 mmol/l benzamidine-HCl. The islets were resuspended (2000 islets/ml) in the same buffer supplemented with 1% (weight/volume, w/v) Trasylol, 5 mmol/l EDTA, 0.1 mmol/l p-chloromercuribenzenesulfonic acid and 1% Triton X-114. The suspension was incubated on ice for 2 h to solubilize the islets and then centrifuged at 100,000 × g for 30 min. The lysate supernatant was used for immunoprecipitation. Detergent lysate was pre-cleared by incubation for 1 h on ice with a pool of normal human serum (5 µl per 100 µl lysate) and binding to protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) for 30 min on ice. After centrifugation, the supernatant was incubated with 10 µl serum for 16 h at 4°C followed by 45 min incubation with 100 µl protein A-Sepharose CL-4B. The immunoprecipitates were washed 12 times by centrifugation in 1 ml of buffer containing 20 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 0.5 mmol/l methionine and 10 mmol/l benzamidine-HCl, 0.5% Triton X-114 and once in ice-cold double distilled water containing 0.02% (w/v) Trasylol. Proteins were eluted from the immunosorbent by boiling for 1 min in 80 mmol/l Tris-HCl (pH 6.8), containing 3% SDS, 15% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.006% (w/v) bromophenol blue. Immunoprecipitated proteins were separated by molecular weight with 8% SDS-PAGE. The gels were processed for fluorography using Amplify (Amersham International) and Konica X-ray film. All fluorographs were analysed independently by at least two observers without knowledge of sample identity. Samples were first ranked positive and negative after comparison with the positive and negative controls run with every experiment. A quantitative estimate of 64K antibody positivity in samples was also obtained by densitometric scanning of bands corresponding to the protein on the fluorograph. Peak height was taken as a measure of band density and results were expressed as a proportion of the height of peaks corresponding to the positive controls in each experiment. The inter-assay coefficient of variation (CV) and the intraassay CV were 15.3% and 7.9%, respectively.

Thyroid function tests

Serum T₄ and free T₄ (FT₄) were measured by RIA kits (Travenol, Co., Ltd., Tokyo, Japan). Serum T₃, free T₃ (FT₃) and TSH were also measured by RIA kits (Eiken Chemical Co., Ltd., Tokyo, Japan). The normal ranges of T₄, FT₄, T₃, FT₃ and TSH are 57.9–148.0 nmol/l, 7.7–29.6 pmol/l, 1.4–2.2 nmol/l, 3.4–10.3 pmol/l and 0.5–5.0 µU/ml, respectively.

Assays of organ-specific autoantibodies

Anti-thyroidal microsomal antibodies (ATMA) and anti-thyroglobulin antibodies (ATGA) were measured by haemagglutination tests using commercial kits (Serodia Amc, Fujirebio Inc., Tokyo, Japan). Anti-adrenal antibodies (AAA) and anti-gastric antibodies (AGA) were measured by indirect immunofluorescence technique. The cutoffs were ×100 for ATMA and ATGA, ×1 for AAA ×10 for AGA.

Statistical analysis

Statistical tests used were Chi-square test and Student's *t*-test. The values are expressed as mean ± SD.

Fig. 1. Fluorograph illustrating proteins immunoprecipitated from detergent lysates of [^{35}S]-methionine labelled rat islets by sera from nine Type 1 (insulin-dependent) diabetic patients. Proteins immunoprecipitated by positive (+ve) and negative (-ve) 64K antibody control sera tested in same experiment are shown at left

Fig. 2. Correlation between levels of 64K antibodies and islet cell antibodies (ICA) titres. Levels of 64K antibodies were quantified by densitometric scanning of the appropriate bands on fluorograph, expressing results relative to the positive control. JDF units, Juvenile Diabetes Foundation units

Results

The frequency of autoantibodies to 64,000- M_r protein in long-term Type 1 diabetes

Autoantibodies to 64,000- M_r protein were determined in 46 Type 1 diabetic patients using at least two separate islet preparations. Figure 1 shows a representative fluorograph illustrating proteins immunoprecipitated from total lysates by sera from nine patients. On this gel samples 1, 2, 3, 4, 6 and 7 were found to be positive for 64K antibodies whereas samples 5, 8 and 9 were negative. Of the 46 Type 1 diabetic patients, 18 (39.1%) patients were positive for 64K antibodies. The clinical characteristics of 18 patients who were positive for 64K antibodies are shown in Table 2. As shown in Table 2, 12 of 18 patients positive for

64K antibodies had AITD (seven Graves' disease and five Hashimoto's thyroiditis) and Graves' disease preceded Type 1 diabetes in cases 4, 5, 8 and 15. Serum C-peptide levels at 3 min after the injection of glucagon were not detected in 15 of these 18 patients. The 64K antibodies were also tested using sera obtained from 10 newly-diagnosed Type 1 diabetic patients and 20 control individuals. None of the control individuals were positive for any organ-specific antibodies. The 64K antibodies were positive in eight (80%) of 10 newly-diagnosed Type 1 diabetes and none of the control individuals (data not shown), a frequency similar to that in earlier studies [7–11].

Correlation between levels of antibodies to the 64,000- M_r protein and titres of ICA

ICA were detected in 15 (32.6%) of 46 patients by an immunoenzymatic method using human pancreatic sections and the ICA range was from 2.5 to 1280 JDF units. All ICA-positive sera reacted with Beta and non-Beta cells in islets by double staining with anti-glucagon antibodies (data not shown). All ICA-positive patients were positive for 64K antibodies, and 12 of the 15 ICA- and 64K-antibody-positive patients had AITD (Table 2). A quantitative estimate of the 64K antibody levels was obtained by densitometric scanning of appropriate bands on the fluorograph, and results expressed relative to the positive control. Using this analysis, we observed a correlation between levels of 64K antibodies and titres of ICA (Fig. 2). As shown in Figure 2, it seems that higher levels of 64K antibodies were observed in the patients who had higher ICA titres, but 64K antibodies were also detected clearly in the patients with a low ICA titre of less than 20 JDF units. There was no correlation however between levels of 64K antibodies with duration of the disease and serum C-peptide levels (data not shown).

Discussion

Type 1 diabetes is frequently associated with AITD, pernicious anaemia and idiopathic Addison's disease. Furthermore, Type 1 diabetic patients without clinical manifestations of organ-specific autoimmune disease and their relatives have a high prevalence of thyroid microsomal, gastric parietal cell and adrenal antibodies [15–18] and these findings support an autoimmune aetiology for Type 1 diabetes. It has been reported that ICA persist in some Type 1 diabetic patients with concomitant autoimmune disease [4–6]. However, there have been no reports regarding the determination of 64K antibodies in long-term Type 1 diabetic patients.

In the present study, 64K antibodies were assayed in Type 1 diabetic patients with a disease duration of more than 5 years in order to investigate the characteristics of Type 1 diabetic patients with long-term diabetes who were positive for antibodies to 64K antigens. The 64K antibodies have been detected in 70–90% of newly-diagnosed Type 1 diabetic patients [7–11] and in some cases have persisted 6–7 years after onset [10]. The autoanti-

Table 2. Clinical characteristics of 18 Type 1 (insulin-dependent) diabetic patients positive for antibodies to the 64,000-M_r islet protein

No.	Sex	Age (years)	Duration of diabetes (years)	AITD	C-peptide ^b (ng/ml)	ICA(JDF units)	ATMA (titres)
1	F	53	13	Hashimoto	0.3	1280	× 25 600
2	M	30	12	Hashimoto	0.3	160	× 6 400
3	M	42	10	Graves	0.3	160	× 25 600
4	F	49	7	Graves ^a	0.6	160	× 25 600
5	F	47	5	Graves ^a	1.2	160	× 409 600
6	F	38	5	Hashimoto	0.3	80	× 409 600
7	F	68	15	(-)	0.3	20	(-)
8	M	32	6	Graves ^a	1.5	20	× 6 400
9	M	48	24	Graves	0.3	2.5	× 400
10	M	33	12	(-)	0.3	2.5	(-)
11	M	15	10	(-)	0.3	2.5	(-)
12	F	26	9	Hashimoto	0.3	2.5	× 6 400
13	F	54	7	Graves	0.3	2.5	× 400
14	F	22	6	Hashimoto	0.3	2.5	× 1 600
15	M	43	6	Graves ^a	0.3	2.5	× 6 400
16	F	22	7	(-)	0.3	negative	(-)
17	F	28	19	(-)	0.3	negative	(-)
18	F	46	19	(-)	0.3	negative	(-)

^a Graves' disease preceded Type 1 diabetes;^b Serum C-peptide level at 3 min after the injection of glucagon. AITD, Autoimmune thyroid disease; Hashimoto, Hashimoto's thyroiditis; Graves, Graves' disease; ICA, islet cell antibodies; JDF units, Juvenile Diabetes Foundation units; ATMA, anti-thyroid microsomal antibodies

bodies to 50 kDa and 37/40 kDa tryptic fragments of the 64K antigen have also been frequently identified in Type 1 diabetic patients and antibodies to 37/40 kDa tryptic fragments have been reported as the predictive marker for diabetes [19, 20]. In this study, 64K antibodies were determined in 18 (39.1%) of 46 Type 1 diabetic patients and 12 of these 18 patients had AITD. On the other hand, ICA were detected in 15 (32.6%) of 46 long-term Type 1 diabetic patients and all of these ICA-positive patients were positive for 64K antibodies, and 12 of these 15 ICA- and 64K-antibody-positive patients had AITD. As for the correlation between levels of 64K antibodies and titres of ICA, higher levels of 64K antibodies were observed in patients with higher titres of ICA. However, the 64K antibodies were also detected clearly in our patients with low ICA titres less than 20 JDF units and in three ICA-negative patients. These results could be interpreted in two ways. Firstly, immunoprecipitation of metabolically radiolabelled endogenous proteins from islets followed by electrophoresis and fluorography have the advantage of having a higher sensitivity compared with the immuno-enzymatic method for ICA assay. An alternative explanation would be that perhaps the 64K antigen, being a minor component of the pancreatic Beta cells, may not have been included in the antigens for ICA. The 64K antigen was recently identified as the γ -aminobutyric acid (GABA)-synthesizing enzyme glutamic acid decarboxylase (GAD) in pancreatic Beta cells [12]. Recently it has been reported by Eisenbarth et al. [21, 22] that there is species and concordant histology heterogeneity in ICA. ICA which are restricted react with human and rat but not mouse islets, specifically, Beta cells. In contrast non-restricted ICA react with human, rat and mouse islets and all

islet cells. They demonstrated that relatives expressing only restricted ICA less often progressed to diabetes, and one autoantigen of the restricted ICA was GAD. In our study, all ICA-positive sera reacted with Beta and non-Beta cells in islets by double staining with anti-glucagon antibodies. From this point of view, it seems that ICA in our patients may be non-restricted ICA and the autoantigen of these ICA may not be GAD. However, an absolute proof that the ICA in our patients do not react with GAD on the pancreas section will require purified or cloned GAD. It should be noted therefore, that the C-peptide levels in almost all patients with persisting positive ICA or 64K antibodies were undetectable despite increased titres of such antibodies. In addition, we have previously reported that ICA could be detected in 65% of ICA-negative Type 1 diabetic patients by using the Epstein-Barr virus transformation and hybridoma technology [14]. This evidence demonstrates that ICA were produced through non-specific stimulation of the B lymphocytes in the peripheral blood of ICA-negative Type 1 diabetic patients. Therefore, these results suggest that the production of ICA or 64K antibodies might be regulated, not only by the amount of residual pancreatic Beta cells but also by abnormalities of the autoimmune system in AITD. In a recent report, it was suggested that polyclonal production of autoantibodies exists in AITD [23]. In our study, almost all of ICA- or 64K-antibody-positive patients had AITD. Therefore, there is a possibility that such persisting positive antibodies might be due to polyclonal activation of the autoimmune system in AITD.

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