表3　2型糖尿病感受性遺伝子にみられる集団差[1~5,11]

遺伝子	集団	オッズ比	p 値	マイナーアレル頻度
TCF7L2	ヨーロッパ人	1.37	1.0×10^{-48}	0.31/0.25
	日本人	1.70	7.0×10^{-4}	0.05/0.02
KCNQ1	ヨーロッパ人	1.29	7.8×10^{-4}	0.03/0.05
	日本人	1.43	2.8×10^{-29}	0.31/0.40

いによる．日本人ではこれが1桁低いために，ヨッローパ系集団と同様なオッズ比を示すものの，2,000人の患者と2,000人の健常者を解析しても明瞭な関連は観察されない．[11]

これとは対照的な状況が *KCNQ1* について見られる．すなわち，日本人では p 値が 10^{-29} と明確な関連が認められ，また韓国人，中国人試料についても同様に明確な関連が認められた一方，ヨーロッパ系では同様なオッズ比が認められるものの，p 値が 10^{-4} レベルにとどまる．[5] すなわち，ヨーロッパ系集団とアジア系集団の各々において代表的な2型糖尿病感受性遺伝子は，いずれも集団差を越えて共通する遺伝要因であるけれども，その頻度が大きく異なるために，それぞれの集団における重要性は異なるといえる．いうまでもなく，前述の非肥満型糖尿病に特徴的な感受性遺伝子 *KCNJ15* もまた，明瞭な集団差を示す典型例といえる．

同様な集団差は，上述したナルコレプシーの新規感受性領域 *CPT1B/CHKB* についても認められた(表4)．[8] すなわち，日本人及び韓国人ではアレル頻度が近似して共に有意な関連が認められたが，ヨーロッパ系アメリカ人及びアフリカ系アメリカ人

表4　ナルコレプシーの感受性遺伝子に見られる集団差[8]

集団	患者数/健常者数	mAF 患者	mAF 健常者	オッズ比	p 値
日本人	381/579	0.251	0.158	1.79	4.4×10^{-7}
韓国人	115/309	0.248	0.191	1.40	0.03
USA ヨーロッパ系	388/397	0.053	0.040	1.33	0.12
USA アフリカ系	86/98	0.047	0.026	1.86	0.14

mAF；マイナーアレル頻度．

ではオッズ比で同じ傾向を示すものの，アレル頻度が低いために有意差には至らなかった．これもまた，それぞれの集団における寄与度が異なる例と考えられる．

このように，2型糖尿病及びナルコレプシーの成果からヨーロッパ系集団だけ大規模に解析していれば，すべての遺伝要因が見いだされるわけではないことが分かる．すなわち，日本人/アジア系集団において重要な遺伝要因の全容を知るためには，日本人/アジア系集団自体の研究が必須であることを教えてくれる．

6　今後の課題

2型糖尿病については，既に世界から20以上の感受性遺伝子が報告されており，そのうちの10個余りは日本人患者においてもリスク要因となっている．[12] これらのリスクアレル間に相加的効果が認められ，リスクアレルを多く持つほどオッズ比が上昇することも分かった．しかしながら，それらは遺伝要因全体の一部しか説明できないことから，GWASを用いる戦略に懐疑的な議論もある．筆者は，この問題提起に答えるにはまだ時期尚早だと考えている．その最大の理由は，ほとんどの研究がまだGWASの可能性を活かし切っていないことにある．例えば，同じ病名のついた患者群の試料をただ集めただけで行ったGWASでは見いだせない感受性遺伝子を，患者毎の臨床情報も注意深く収集し臨床的亜型に着目した解析を行うことで，見いだせる可能性が大きいと考えている．前述した非肥満型糖尿病の感受性遺伝子 *KCNJ15* は，その典型例である．むしろ，ありふれた病気をその遺伝素因から見れば，互いに類似するが異質性もある多くの病気の集合体と見るのが自然ではないかと思う．また，欧米のGWASによく見られるように，統計学的な検出力だけを考慮して圧倒的な数の試料を各地からかき集めた結果，地域集団間の異質性(階層化)がいわばノイズとなって，真の感受性遺伝子を発見し難くしている状況もあると考える．

さらには感受性遺伝子多型の多くが，いわゆるゲノムワイド有意水準には到達せず中間的な p 値を

示していると考えられることから，それらの“gray zone”から真の感受性遺伝子多型を同定する方法の確立が，今後解決すべき大きな課題といえる．GWASをスクリーニング段階ととらえ，遺伝子アノテーション，パスウェイ情報や他分野からの知見を組み合わせることも必要であろう．

見いだされた複数の感受性遺伝子が，特定のパスウェイあるいはネットワークに帰属することが分かれば，疾患の発症・病態形成の機序を解明し，新たな治療法を開発するための極めて有用なヒントになる．このことは，遺伝要因の全容が明らかになる以前に，期待できる大きな成果であるといえる．

7 おわりに

疾患関連遺伝子の探索について，ゲノム全域に分布するSNPを利用する戦略の現状と課題を解説した．一方，現在急速な発展を見せている次世代シークエンサーを用いたリシークエンス技術は，初めは家系内伝達が明瞭な，あるいは低頻度ながらオッズ比が高い変異を同定するのに用いられ，徐々に頻度の高い疾患関連多型の検出にも用いられていくであろう．この新技術から生み出される膨大なデータから，良質の望ましい情報を取り出すシステムの確立も今後の大きな課題である．

なお我々は，疾患関連遺伝子の同定を促進するために「統合データベースプロジェクト」のなかでGWASデータベースを構築している[13]（https://gwas.lifesciencedb.jp/index.Japanese.html）．なるべく多くのGWAS結果を受け入れ，個人特定につながらない情報を公開し，より詳細な情報を研究者の申請に応じて提供することによって，更に多数の疾患関連遺伝子が特定され，発症・病態機序の理解が進むことを期待している．

文　献

1) Sladek R. *et al*., *Nature*, 445, 881–885 (2007).
2) Saxena R. *et al*., *Science*, 316, 1331–1336 (2007).
3) Scott L. J. *et al*., *Science*, 316, 1341–1345 (2007).
4) Wellcome Trust Case Control Consortium, *Nature*, 447, 661–678 (2007).
5) Yasuda K. *et al*., *Nat. Genet*., 40, 1092–1097 (2008).
6) Unoki H. *et al*., *Nat. Genet*., 40, 1098–1102 (2008).
7) Okamoto K. *et al*., *Am. J. Hum. Genet*., 86, 54–64 (2010).
8) Miyagawa T. *et al*., *Nat. Genet*., 40, 1324–1328 (2008).
9) Hallmayer J. *et al*., *Nat. Genet*., 41, 708–711 (2009).
10) Tanaka Y. *et al*., *Nat. Genet*., 41, 1105–1109 (2009).
11) Miyake K. *et al*., *J. Hum. Genet*., 53, 174–180 (2008).
12) Miyake K. *et al*., *J. Hum. Genet*., 54, 236–241 (2009).
13) Koike A. *et al*., *J. Hum. Genet*., 54, 543–546 (2009).

Clinical Immunology (2011) 138, 146–153

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

Differences in the humoral autoreactivity to zinc transporter 8 between childhood- and adult-onset type 1 diabetes in Japanese patients

Eiji Kawasaki[a,*], Kan Nakamura[a], Genpei Kuriya[b], Tsuyoshi Satoh[b], Masakazu Kobayashi[b], Hironaga Kuwahara[b], Norio Abiru[b], Hironori Yamasaki[c], Nobuo Matsuura[d], Junnosuke Miura[e], Yasuko Uchigata[e], Katsumi Eguchi[b]

[a] *Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, Nagasaki, Japan*
[b] *Department of Endocrinology and Metabolism, Unit of Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan*
[c] *Center for Health and Communicating Medicine, Nagasaki University, Nagasaki, Japan*
[d] *Department of Early Childhood Education, Seitoku University, Matsudo, Japan*
[e] *Diabetes Center, Tokyo Women's Medical University School of Medicine, Tokyo, Japan*

Received 23 July 2010; accepted with revision 13 October 2010
Available online 10 November 2010

KEYWORDS
Autoantibodies;
Epitope;
Fulminant;
HLA;
Type 1 diabetes;
Zinc transporter-8

Abstract The aim of this study was to evaluate the humoral autoreactivity to zinc transporter 8 (ZnT8) depending on the clinical phenotype of type 1 diabetes (T1D). ZnT8 autoantibodies (ZnT8A) were determined by radioimmunoassay using carboxy-terminal ZnT8 constructs in 57 childhood-onset, 97 adult-onset, and 85 fulminant T1D. The ZnT8A frequency was higher in childhood-onset patients and decreased with increasing age of onset from 70% to 24% ($P_{trend}<0.005$). None of the patients with fulminant T1D was positive for ZnT8A. There were at least two distinct ZnT8A epitope patterns associated with the aa325-restriction, childhood-onset patients have aa325-nonrestricted response more frequently compared to the adult-onset group ($P<0.05$). The level of ZnT8A was inversely associated with the copy number of HLA-*DR4* allele ($P<0.05$). These results suggest differences in the humoral autoreactivity to ZnT8 depending on the clinical phenotype, which should provide strategy for autoantibody measurement in subjects to allow early diagnosis of autoimmune T1D.

* Corresponding author. Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Fax: +81 95 819 7552.
E-mail address: eijikawa@nagasaki-u.ac.jp (E. Kawasaki).

1521-6616/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.clim.2010.10.007

1. Introduction

Type 1 diabetes is an autoimmune disease characterized by T-cell-mediated destruction of pancreatic β cells and the presence of circulating autoantibodies directed against several β cell autoantigens [1]. Although type 1 diabetes is frequently considered to be a childhood disease, it may develop at any age, and a greater proportion of type 1 diabetic cases are diagnosed later in life [2]. Moreover, there is increasing evidence that type 1 diabetes, especially in adult-onset patients, includes clinically and immunologically heterogeneous type. Those include slow-onset and fulminant type 1 diabetes [3]. Although the different clinical phenotypes may depend on the extent of β cell destruction, the underlying immune mechanisms are largely unknown.

To date, the expression of anti-islet autoantibodies has been the best phenotypic marker of autoimmune type 1 (type 1A) diabetes [1]. Recently, the cation efflux transporter zinc transporter 8 (ZnT8) has been identified as a novel target autoantigen in patients with type 1 diabetes [4]. Zinc transporters are multipass transmembrane proteins that function in the transport of zinc out of the cytoplasm or into the vesicles [5]. ZnT8 is specifically expressed in the pancreatic β-cells and plays a major role in insulin maturation [4,6]. Previous studies have reported that autoantibodies to ZnT8 (ZnT8A) were identified in more than 60% of young patients with type 1 diabetes and the combined measurement of autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), and protein tyrosine phosphatase IA-2 (IA-2A), and ZnT8A raised autoimmunity detection rates to 98% at disease onset in Caucasoid populations [4]. However, the relevance of ZnT8A in patients with adult-onset type 1 diabetes, especially in cases of slow-onset and fulminant type 1 diabetes, has not been clarified. The intent of this study was to evaluate the association of humoral autoreactivity to ZnT8 with clinical heterogeneity in Japanese patients with type 1 diabetes and establish its potential use as an additional marker of autoimmunity and phenotype characterization. We also examined the influence of HLA-*DR* on reactivities to ZnT8 protein.

2. Materials and methods

2.1. Subjects

One hundred and sixty-six new-onset patients with type 1 diabetes consecutively recruited at our hospital between 1982 and 2008 with disease duration <6 months were studied. They consisted of 57 childhood-onset patients (childhood-onset, age <15 years) (59.7% female, age 9.7 ± 3.6, median 10.0, range 2.0–14.0 years, median duration 0.40, range 0–6.0 months) and 97 adult-onset patients (adult-onset, age ≥18 years) (63.9% female, age 35.1 ± 16.2, median 28.0, range 18.0–77.0 years, median duration 0.45, range 0–6.0 months) with type 1 diabetes. The remaining 12 patients with type 1 diabetes diagnosed between 15 and 17 years of age (58.3% female) were unclassified.

Adult-onset subjects were further divided into three groups according to the mode of diabetes onset (acute-onset, slow-onset, and fulminant). In patients with acute-onset type 1 diabetes, the duration of hyperglycemic symptoms before the start of insulin therapy was less than 3 months. In patients with slow-onset type 1 diabetes, insulin treatment was initiated >1 year after the diagnosis of diabetes by the positive urine glucose test or the development of hyperglycemic symptoms [7]. Diagnostic criteria for fulminant type 1 diabetes were 1) ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms, 2) plasma glucose level ≥16 mM and HbA1c <8.5% at the first visit, and 3) urinary C-peptide level <10 μg/day, fasting serum C-peptide level <0.3 ng/ml or serum C-peptide <0.5 ng/ml after glucagon or a meal load [3]. Of 97 adult-onset patients, 54 (55.7%) were acute-onset, 28 (28.9%) were slow-onset, and 15 (15.5%) had fulminant type 1 diabetes. To increase the number of patients in this study, we also examined the data for a second set of patients with fulminant type 1 diabetes (n=70), which was provided by the Fulminant Type 1 Diabetes Committee of the Japan Diabetes Society [8]. Therefore, a total of 85 patients with fulminant type 1 diabetes (36.5% female, age 43.3±16.1 years) were used for autoantibody analysis. All childhood-onset subjects were considered to be acute-onset forms based on the aforementioned criteria. All patients with diabetes analyzed in the present study were diagnosed according to the American Diabetes Association criteria for the classification of diabetes [9]. All subjects were informed of the purpose of the study, and their consent for study participation was obtained. Protocols were approved by the ethics committee of Nagasaki University and the Japan Diabetes Society. Sera were stored at −20 °C until use.

2.2. ZnT8 autoantibody assay

Fig. 1 illustrates the secondary structure of full-length human ZnT8 and the constructs used in this study. ZnT8A were determined by radioligand binding assay using a dimeric cDNA construct of the carboxy-terminal domains (aa268–369) carrying 325Trp and 325Arg (CW-CR), which showed higher sensitivity with the same specificity compared with individual monomeric constructs in our previous study [10]. The cut-off value for ZnT8A-CW-CR was an index of 0.007, which was based on the 99th percentile of sera from 139 healthy control subjects. The inter-assay coefficient of variation (CV) and intra-assay CV values were 9.6% and 4.6%, respectively. In this study, ZnT8A were considered as "positive" if sera were ranked as positive for ZnT8A-CW-CR. In the Diabetes Autoantibody Standardization Program 2009 (DASP 2009), this assay had 40% sensitivity and 100% specificity.

Autoantibody reactivities to ZnT8 aa325 variants were also determined using the carboxy-terminal domains (aa268–369) of cDNA encoding the aa325 codon variants CCG (Arg, CR), TCG (Trp, CW), and CAG (Gln, CQ) to analyze an epitope specificity (Fig. 1). The cut-off index was 0.018 for ZnT8A-CW, 0.016 for ZnT8A-CR, and 0.006 for ZnT8A-CQ based on the 99th percentile of sera from 139 healthy control subjects. The inter-assay CV and intra-assay CV values were 5.9% and 6.8% (ZnT8A-CW), 10.4% and 5.7% (ZnT8A-CR) and 6.3% and 7.5% (ZnT8A-CQ), respectively. These autoantibodies were determined in 52 childhood-onset and 161 adult-onset patients, including 85 patients with fulminant type 1 diabetes because of serum availability.

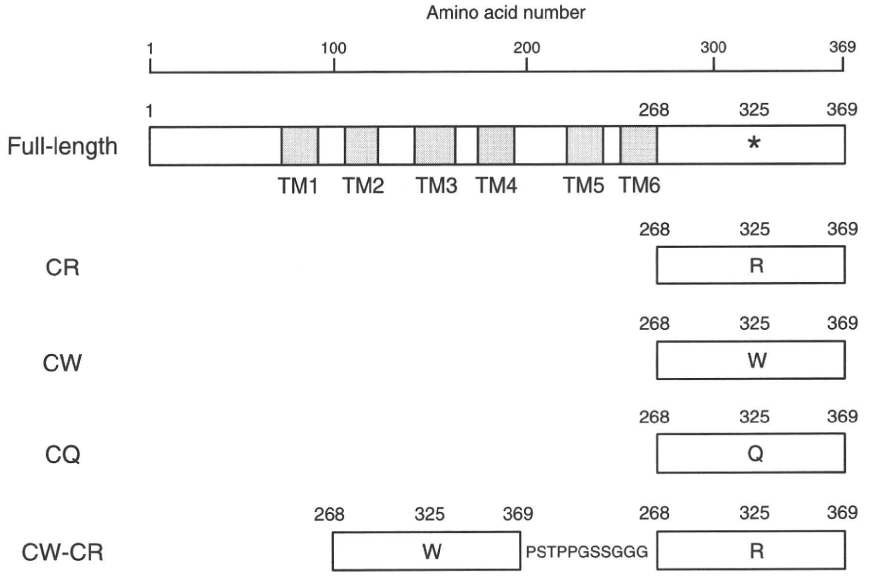

Figure 1 Schematic representation of full-length human ZnT8 and the amino acid boundaries of four constructs used in this study. Numbers correspond to the amino acid residues of the ZnT8 published sequence [4,6]. A hinge sequence of CW-CR construct is derived from the human IgG heavy chain. TM, transmembrane region; *polymorphic site.

2.3. Detection of other anti-islet autoantibodies

We used a radioligand binding assay to detect GADA and IA-2A using the cDNA for full-length human islet GAD65 and the complete cytoplasmic region of IA-2 (aa601–979), respectively, as previously described [11]. "Positive" was based on the 99th percentile of sera from 204 healthy control subjects without family history of diabetes. The cut-off indices were 0.028 for GADA and 0.018 for IA-2A. The inter-assay CV and intra-assay CV were 3.3% and 5.3% for GADA and 1.9% and 2.1% for IA-2A, respectively. In the DASP 2005, the GADA and IA-2A assays had sensitivities of 74% and 68% and specificities of 98% and 96%, respectively.

The insulin autoantibody (IAA) assay was carried out by a micro-IAA assay format as previously described [12]. Based on the difference in cpm between wells without and with cold insulin, an index was determined, with a positivity criterion of 0.010 based on the 99th percentile of sera from healthy control subjects. The inter-assay CV and intra-assay CV were 6.8% and 1.4%, respectively. In the DASP 2005, this assay had a sensitivity of 58% and a specificity of 98%. IAA were determined in sera obtained within 2 weeks after initiation of insulin therapy.

2.4. HLA typing

HLA-*DR* typing was performed by a standard microcytotoxicity test or PCR-amplified DNA and nonradioactive sequence-specific oligonucleotide probes [11].

2.5. Statistical analysis

Results were expressed as the mean±SD unless otherwise indicated. Autoantibody prevalence was compared using the Chi-square test, Fisher's exact test, and Cochran–Armitage's test where appropriate. Differences in nonparametric data were tested by the Mann–Whitney *U* test or the Kruskal–Wallis test. Comparisons of the ZnT8A levels were made by ANOVA with HLA-DR allele alone and ANOVA with the HLA-DR allele and phenotypic group (childhood-onset and adult-onset). The correlation between autoantibody levels was analyzed using the Spearman rank correlation test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Humoral autoreactivity to a hybrid ZnT8 construct

ZnT8A (ZnT8A-CW-CR) were detected in 33 of 57 (58%) childhood-onset patients with type 1 diabetes, which was significantly higher than that in the adult-onset group (33 of 97, 34%, $P=0.004$). However, the level of ZnT8A in patients positive for ZnT8A was similar between the two groups (childhood-onset group, median index=0.088, range 0.010–0.606; adult-onset group, median index=0.067, range 0.009–0.669, $P=0.42$). The prevalence of ZnT8A with respect to onset age was also evaluated after the combination of the two groups. Childhood-onset and adult-onset patients were combined and divided according to the age of onset into four groups (ages <10, 10–14, 18–30, and >30 years); the prevalence of ZnT8A was then evaluated by Cochran–Armitage's trend test. The prevalence of ZnT8A was inversely related to the onset age (70%, 50%, 41%, and 24%, respectively, $P=0.004$). In the adult-onset group, acute-onset patients had a higher frequency of ZnT8A than did slow-onset patients (50% vs. 21%, $P=0.012$). However, none of the 85 patients with fulminant type 1 diabetes was positive for ZnT8A. There was no statistical difference between patients' gender and the prevalence or level of ZnT8A (data not shown).

Table 1 shows the clinical and immunogenetic characteristics between ZnT8A-positive and -negative patients with non-fulminant type 1 diabetes. In the childhood-onset group, GADA ($P<0.005$), IA-2A ($P<0.0001$) and IAA ($P<0.05$) were

Table 1 Comparisons of clinical and immunogenetic features between type 1 diabetic patients with and without ZnT8A.

	Childhood-onset				*P* value[a]	Adult-onset				*P* value[a]
	n	ZnT8A+ve	n	ZnT8A−ve		n	ZnT8A+ve	*n*	ZnT8A−ve	
Male, *n* (%)	33	14 (42)	24	10 (42)	NS	33	8 (24)	49	18 (37)	NS
Age at onset (years)	33	9.1±3.8	24	10.6±3.3	NS	33	32.5±16.3	49	36.1±17.5	NS
GADA+ve, *n* (%)	33	30 (91)	24	13 (54)	<0.005	33	27 (82)	49	37 (76)	NS
IA-2A+ve, *n* (%)	33	31 (94)	24	10 (42)	<0.0001	33	21 (64)	49	13 (27)	<0.001
IAA+ve, *n* (%)[b]	26	16 (62)	15	4 (27)	<0.05	26	18 (69)	39	20 (51)	NS
HLA-*DR4*+ve, *n* (%)	21	12 (57)	19	12 (63)	NS	27	17 (63)	44	24 (55)	NS
HLA-*DR9*+ve, *n* (%)	21	11 (52)	19	10 (53)	NS	27	14 (52)	44	18 (41)	NS

Patients with fulminant type 1 diabetes were excluded from this analysis.
Data are means±SD or *n* (%); NS, not significant.
[a] χ^2 test for proportions; Mann–Whitney *U* test for continuous data.
[b] IAA were evaluated in sera which were obtained within 2 weeks after initiating insulin therapy.

positive at higher proportions in the ZnT8A-positive than ZnT8A-negative patients. However, only the prevalence of IA-2A in ZnT8A-positive patients was significantly higher than that in ZnT8A-negative patients in the adult-onset group ($P<0.0001$). There was no correlation between the ZnT8A positivity and the prevalence of two major susceptible class II HLA alleles in the Japanese, *DR4* and *DR9*, in either group. HLA-*DR9* was associated with the presence of IA-2A in our patients (Supplementary Table 1).

3.2. Humoral autoreactivity to ZnT8 aa325 variants

We and others reported that the amino acid encoded by the polymorphic codon 325 is a key determinant and there are three classes of conformational epitopes: one for which 325Arg is an essential determinant, a second that is 325Trp-restricted, and a third that is not affected by aa325 [10,13]. Therefore, to assess the possible difference on the ZnT8A epitope recognition between childhood-onset and adult-onset patients, we also tested sera for the reactivity to the carboxy-terminal ZnT8 constructs bearing 325Trp (CW), 325Arg (CR), or 325Gln (CQ).

In the childhood-onset group, 29 of 52 (56%) patients reacted to at least one construct, with the highest response recorded in reaction to the CW construct (44%) followed by the CR (38%) and CQ (31%) constructs (Fig. 2). An analysis of the overlap in responses shows that 6% and 12% of patients reacted to the CR or CW construct alone, respectively, and rarely to the CQ construct alone (2%); 21% of patients reacted to all three constructs. In the adult-onset group, 24 of 75 (32%) patients reacted to at least one construct, which was significantly lower than the occurrence in the childhood-onset group ($P=0.008$). This difference fundamentally results from patients who reacted to all three constructs. The prevalence of patients with 325Trp- or 325Arg-restricted response was similar between the two groups. However, the proportion of patients who had ZnT8A not affected by aa325 (aa325-nonrestricted ZnT8A) was frequent in the childhood-onset group among patients who reacted to at least one construct (38% vs. 13%, $P<0.05$). None of the 85 patients with fulminant type 1 diabetes reacted with any of the ZnT8 variant constructs.

3.3. ZnT8A titer and class II HLA

It has been reported that HLA characteristics were associated with the frequencies and levels of anti-islet autoantibodies

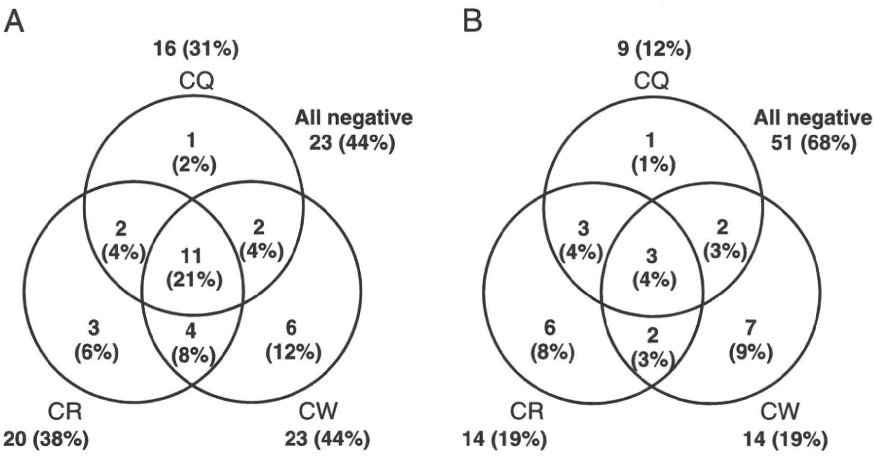

Figure 2 Humoral autoreactivity to 325Trp (CW), 325Arg (CR), and 325Gln (CQ) constructs in patients with childhood-onset (A) and adult-onset type 1 diabetes (B). Venn diagram illustrates the overlap of autoantibody detection with each of the polymorphic construct in 52 childhood-onset and 75 adult-onset patients with type 1 diabetes.

[14,15]. We therefore examined the association between ZnT8A and HLA-DR. Although there were no associations between the positivity of ZnT8A and the frequency of the HLA-*DR4* or *DR9* allele in our subjects, the level of ZnT8A was associated with the copy number of the HLA-*DR4* allele. Among the ZnT8A-positive patients, the mean index of ZnT8A in HLA-*DR4* homozygotes (0.041 ± 0.040, mean ± SD) was significantly lower than those in patients carrying no (0.163 ± 0.165) or one copy (0.132 ± 0.097) of *DR4* allele ($P=0.028$ by the Kruskal–Wallis test) (Fig. 3A). HLA-*DR9* had no influence on the level of ZnT8A (Fig. 3B). A mixed model ANOVA using the HLA-*DR4* allele (4/4, 4/X, X/X) and phenotypic group (childhood-onset and adult-onset) as factorial fixed effects revealed no differences in ZnT8A levels between phenotypic groups ($P=0.82$) or phenotype/allele interactions ($P=0.58$).

3.4. Overlapping prevalence with other anti-islet autoantibodies

Fig. 4 illustrates an overlapping prevalence of ZnT8A, GADA, IA-2A, and IAA in patients whose sera were obtained within 2 weeks after the initiation of insulin treatment. The prevalence of GADA, IA-2A, IAA, and ZnT8A was 83%, 78%, 49%, and 61% in the childhood-onset group, and 80%, 41%, 57%, and 39% in the adult-onset group, respectively (Figs. 4A and B), while that for patients with fulminant type 1 diabetes was 9%, 4%, 6%, and 0%, respectively (Fig. 4C). In the childhood-onset group, the combined analysis of GADA and IA-2A revealed type 1A diabetes in 90% of patients (37 of 41) (Fig. 4A). Inclusion of the IAA and/or ZnT8A did not affect the number who tested positive for at least one of these autoantibodies.

In the adult-onset group, the prevalence of patients positive for GADA and/or IA-2A was 89% (54 of 61), and inclusion of the IAA and/or ZnT8A reduced the number of autoantibody-negative subjects from 12% to 5%. Two individuals (40%) from a group of 5 patients who were negative for GADA, IA-2A, and IAA were ZnT8A positive. Of note, the prevalence of patients positive for all four autoantibodies was greater in the childhood-onset group (37%) than that in the adult-onset group (11%, $P=0.003$). On the other hand, the prevalence of one or two autoantibody-positive patients was significantly higher in the adult-onset group (52%) as compared with the childhood-onset group (24%, $P=0.005$). In fulminant type 1 diabetes, most patients were single-autoantibody-positive and only one patient showed an overlap of positivity for GADA and IAA (Fig. 4C).

Analyzed in terms of the levels of autoantibodies, ZnT8A correlated with IA-2A in the childhood-onset patients ($r=0.434$, $P<0.005$) but not in the adult-onset patients ($r=0.056$, $P=0.67$). There was no correlation between levels of ZnT8A with those of GADA or IAA in either group (data not shown).

4. Discussion

We demonstrated 1) different humoral autoreactivity to ZnT8 between adult-onset and childhood-onset type 1 diabetes, 2) an inverse association between the copy number of HLA-*DR4* and the levels of ZnT8A, and 3) no humoral autoreactivity to the ZnT8 molecule in fulminant type 1 diabetes.

The prevalence of ZnT8A was significantly higher in childhood-onset patients than that in adult-onset patients. Furthermore, the prevalence of ZnT8A was inversely related to the onset age with the highest prevalence of 70% in patients aged <10 years. Thus, ZnT8A exhibit heterogeneity with regard to the age of diabetes onset and are good markers of childhood-onset type 1 diabetes. Notably, the higher frequency of ZnT8A in childhood-onset patients fundamentally resulted from an increased number of patients with aa325-nonrestricted ZnT8A (Fig. 2). We and

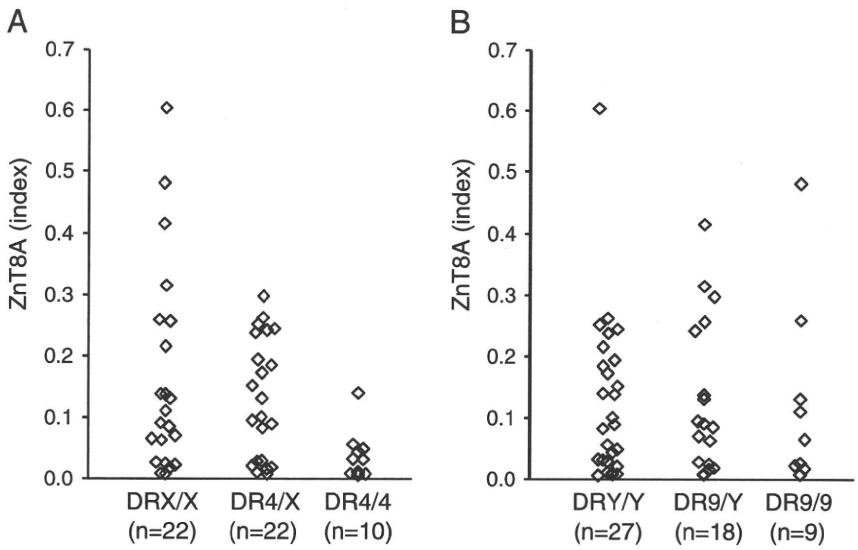

Figure 3 Comparisons of the level of ZnT8A with the copy number of the HLA-*DR4* (A) and -*DR9* (B). "X", non*DR4* allele; "Y", non*DR9* allele (B). The levels were compared among the ZnT8A-positive individuals. The mean (±SD) index of ZnT8A is 0.041 (±0.040) for HLA-*DR4* homozygotes, 0.132 (±0.097) for *DR4/X*, and 0.163 (±0.165) for *DRX/X* ($P=0.043$ by ANOVA). The mean index is 0.127 (±0.155) for HLA-*DR9/9*, 0.130 (±0.124) for *DR9/Y*, and 0.127 (±0.129) for *DRY/Y* ($P=0.99$).

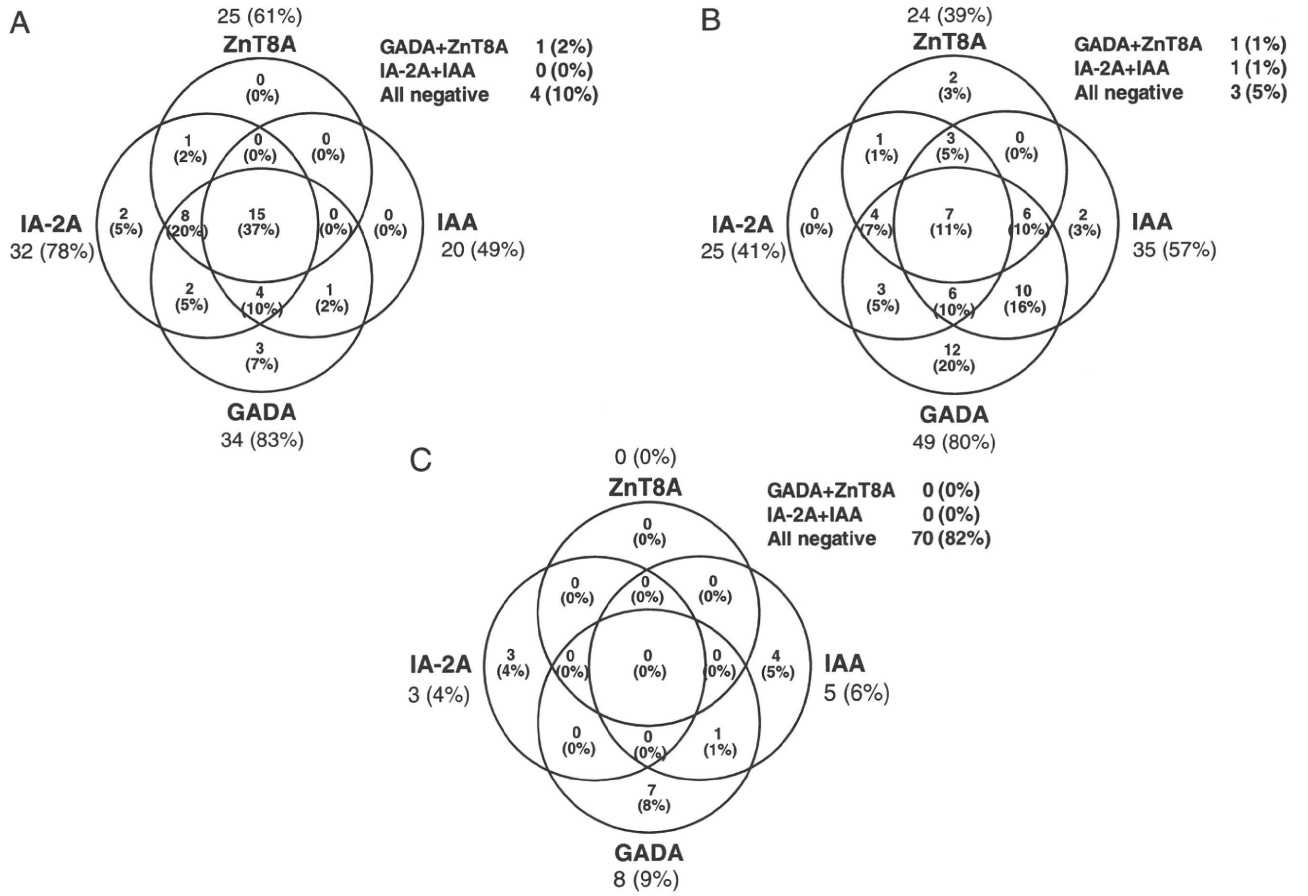

Figure 4 Combinatorial analysis of autoantibodies to ZnT8, GAD65, insulin, and IA-2. A, Childhood-onset type 1 diabetes (n=41); B, adult-onset type 1 diabetes (n=61); C, fulminant type 1 diabetes (n=85). Patients' sera obtained within two weeks after the initiation of insulin treatment were used.

others recently reported that the amino acid encoded by the polymorphic codon 325 (Arg, Trp, Gln) is a key determinant of humoral autoreactivity to this protein [10,13]. Furthermore, Wenzlau and coworkers reported that the C-terminal domain of ZnT8 contains at least three discrete conformational epitopes: 325Trp-restricted, 325Arg-restricted, and aa325-nonrestricted epitopes [10,13]. The considerably higher proportion of subjects with aa325-nonrestricted ZnT8A among childhood-onset patients could be because autoreactivity to ZnT8 reflects a more severe β cell destruction leading to manifestation of the disease early in life, or because the humoral autoreactivity to other cytoplasmic epitopes of ZnT8 is relatively rare in patients who develop type 1 diabetes at an older age.

It has been reported that the HLA characteristics were associated with the frequencies and levels of anti-islet autoantibodies in Caucasoid patients [14,15]. In the present study, we demonstrated that the copy number of HLA-*DR4* is associated with the ZnT8A production (Fig. 3). Furthermore, this association was independent of the clinical phenotype. This novel observation of the HLA-non*DR4* bias of ZnT8A production is one of the interesting findings in this study and is contrary to the previous observations that the level of IA-2A was associated with the HLA-*DR4* allele [14,16]. This may indicate that ZnT8 peptides are poorly presented by *DR4* class II molecules to the T-cell receptors. Analysis of peptide binding to *DR4*, peptide elution studies from the *DR4* homozygote, or visualization of *DR4*-peptide binding interaction will be important to test the possibility of reduced or profound binding. Furthermore, this observation needs to be validated in the Caucasoid population, because type 1 diabetes-susceptible HLA-*DR4* in Japanese patients (*DRB1**0405) is different from that in Caucasoid patients (*DRB1**0401).

Measurement of a combination of autoantibody markers has been suggested as a useful tool for determining type 1A diabetes. However, the clinical utility of ZnT8A might be limited over testing GADA, IA-2A, and IAA in childhood-onset patients. In the present cohort, 90% of the childhood-onset patients were positive for GADA and/or IA-2A, but inclusion of IAA and/or ZnT8A did not increase the sensitivity for identifying type 1A diabetes (Fig. 4). Furthermore, GADA, IA-2A, and IAA were positive in a greater proportion of the ZnT8A-positive patients in the childhood-onset group (Table 1). In the adult-onset group, inclusion of the ZnT8A reduced the number of autoantibody-negative subjects from 8% to 5% and 2 of 5 (40%) patients who were negative for GADA, IA-2A, and IAA were ZnT8A positive. Furthermore, the prevalence of patients positive for one or two of these four autoantibodies was greater in the adult-onset group as

compared with the childhood-onset group ($P<0.005$). Such a broader autoantibody response in adult-onset patients implicates that different pathogenic mechanisms may be involved between adult-onset and childhood-onset type 1 diabetes.

Finally, we also demonstrated that none of the sera from patients with fulminant type 1 diabetes reacted to ZnT8A, although ZnT8A are apparently markers for acute-onset patients with type 1 diabetes. Fulminant type 1 diabetes is a subtype of type 1 diabetes characterized by extremely rapid onset with nearly normal HbA1c level, frequent flu-like symptoms just before the disease onset, and virtually no C-peptide secretion at disease onset [8]. Although the underlying pathogenesis of fulminant type 1 diabetes has not been fully clarified, there are increasing evidence to support the involvement of autoimmune mechanisms [17–19]. However, in contrast to type 1A diabetes, both α and β cells are greatly reduced in number and there is a lymphocytic infiltration in the exocrine pancreas tissue in patients with fulminant type 1 diabetes [20,21]. Furthermore, it has been recently reported that ZnT8A titer declined similarly to C-peptide response after the onset of type 1 diabetes [22], although anti-islet autoantibodies are considered to be an epiphenomenon resulting from the autoimmune destruction of the β cells. Taken together, our findings suggest that ZnT8A might be more specific markers of autoimmune-mediated β cell destruction and that non-autoimmune mechanisms such as antivirus immunity following viral infection of β cells are the major causes of fulminant type 1 diabetes.

In conclusion, our present data demonstrated the differences in the humoral autoreactivity to ZnT8 between adult- and childhood-onset type 1 diabetes, and the non*DR4* bias of the ZnT8A production. Furthermore, clinical phenotypes of Japanese type 1 diabetes are associated with the appearance of different autoantibodies, which should provide a strategy for autoantibody measurement in subjects to promote the early diagnosis of type 1A diabetes.

Supplementary materials related to this article can be found online at doi:10.1016/j.clim.2010.10.007.

Acknowledgments

This study was partly supported by a grant from the Ministry of Education, Culture, Science, Sports and Technology of Japan. We thank the Fulminant Type 1 Diabetes Committee of the Japan Diabetes Society for providing the precious sera from patients with fulminant type 1 diabetes. Vector pJH4.1 and BUN-E antiserum were kindly provided by Dr. John Hutton, Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, CO, USA.

References

[1] E. Kawasaki, R.G. Gill, G.S. Eisenbarth, Type 1 diabetes mellitus, in: G.S. Eisenbarth (Ed.), Molecular Mechanisms of Endocrine and Organ Specific Autoimmunity, R.G. Landes Company, Austin, Texas, 1999, pp. 149–182.

[2] M. Laakso, K. Pyorala, Age at onset and type of diabetes, Diab. Care 8 (1985) 114–117.

[3] E. Kawasaki, N. Matsuura, K. Eguchi, Type 1 diabetes in Japan, Diabetologia 49 (2006) 828–836.

[4] J.M. Wenzlau, K. Juhl, L. Yu, O. Moua, S.A. Sarkar, P. Gottlieb, M. Rewers, G.S. Eisenbarth, J. Jensen, H.W. Davidson, J.C. Hutton, The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes, Proc. Natl Acad. Sci. USA 104 (2007) 17040–17045.

[5] T. Kambe, Y. Yamaguchi-Iwai, R. Sasaki, M. Nagao, Overview of mammalian zinc transporters, Cell. Mol. Life Sci. 61 (2004) 49–68.

[6] F. Chimienti, S. Devergnas, A. Favier, M. Seve, Identification and cloning of a β-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules, Diabetes 53 (2004) 2330–2337.

[7] Y. Sera, E. Kawasaki, N. Abiru, M. Ozaki, T. Abe, H. Takino, H. Kondo, H. Yamasaki, Y. Yamaguchi, S. Akazawa, S. Nagataki, Y. Uchigata, N. Matsuura, K. Eguchi, Autoantibodies to multiple islet autoantigens in patients with abrupt onset type 1 diabetes and diabetes diagnosed with urinary glucose screening, J. Autoimmun. 13 (1999) 257–265.

[8] A. Imagawa, T. Hanafusa, Y. Uchigata, A. Kanatsuka, E. Kawasaki, T. Kobayashi, A. Shimada, I. Shimizu, T. Toyoda, T. Maruyama, H. Makino, Fulminant type 1 diabetes: a nationwide survey in Japan, Diab. Care 26 (2003) 2345–2352.

[9] The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, Report of the expert committee on the diagnosis and classification of diabetes mellitus, Diab. Care 20 (1997) 1183–1197.

[10] E. Kawasaki, M. Uga, K. Nakamura, G. Kuriya, T. Satoh, K. Fujishima, M. Ozaki, N. Abiru, H. Yamasaki, J.M. Wenzlau, H. W. Davidson, J.C. Hutton, K. Eguchi, Association between anti-ZnT8 autoantibody specificities and SLC30A8 Arg325Trp variant in Japanese patients with type 1 diabetes, Diabetologia 51 (2008) 2299–2302.

[11] E. Kawasaki, Y. Sera, N. Fujita, M. Yamauchi, M. Ozaki, T. Abe, K. Yamakawa, S. Uotani, H. Takino, H. Yamasaki, Y. Yamaguchi, Y. Uchigata, N. Matsuura, K. Eguchi, Association between IA-2 autoantibody epitope specificities and age of onset in Japanese patients with autoimmune diabetes, J. Autoimmun. 17 (2001) 323–331.

[12] E. Kawasaki, K. Nakamura, G. Kuriya, T. Satoh, H. Kuwahara, M. Kobayashi, N. Abiru, H. Yamasaki, K. Eguchi, Autoantibodies to insulin, insulinoma-associated antigen-2, and zinc transporter 8 improve the prediction of early insulin requirement in adult-onset autoimmune diabetes, J. Clin. Endocrinol. Metab. 95 (2010) 707–713.

[13] J.M. Wenzlau, Y. Liu, L. Yu, O. Moua, K.T. Fowler, S. Rangasamy, J. Walters, G.S. Eisenbarth, H.W. Davidson, J.C. Hutton, A common non-synonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes, Diabetes 57 (2008) 2693–2697.

[14] E. Sabbah, K. Savola, P. Kulmala, H. Reijonen, R. Veijola, P. Vahasalo, J. Karjalainen, J. Ilonen, H.K. Akerblom, M. Knip, Disease-associated autoantibodies and HLA-DQB1 genotypes in children with newly diagnosed insulin-dependent diabetes mellitus (IDDM). The Childhood Diabetes in Finland Study Group, Clin. Exp. Immunol. 116 (1999) 78–83.

[15] J.P. Wang, Z.G. Zhou, J. Lin, G. Huang, C. Zhang, L. Yang, Y. Yuan, H.F. Zhou, M. Zhou, C. Hou, W.D. Zhou, H. Peng, W.A. Hagopian, Islet autoantibodies are associated with HLA-DQ genotypes in Han Chinese patients with type 1 diabetes and their relatives, Tissue Antigens 70 (2007) 369–375.

[16] K. Savola, E. Bonifacio, E. Sabbah, P. Kulmala, P. Vahasalo, J. Karjalainen, E. Tuomilehto-Wolf, J. Merilainen, H.K. Akerblom, M. Knip, IA-2 antibodies—a sensitive marker of IDDM with clinical onset in childhood and adolescence. Childhood Diabetes in Finland Study Group. Diabetologia 41 (1998) 424–429.

[17] A. Imagawa, T. Hanafusa, Y. Uchigata, A. Kanatsuka, E. Kawasaki, T. Kobayashi, A. Shimada, I. Shimizu, T. Maruyama, H. Makino, Different contribution of class II HLA in fulminant and typical autoimmune type 1 diabetes mellitus, Diabetologia 48 (2005) 294–300.
[18] S. Tanaka, T. Kobayashi, T. Momotsu, A novel subtype of type 1 diabetes mellitus, N Engl J. Med. 342 (2000) 1835–1837.
[19] R. Kotani, M. Nagata, A. Imagawa, H. Moriyama, H. Yasuda, J. Miyagawa, T. Hanafusa, K. Yokono, T lymphocyte response against pancreatic beta cell antigens in fulminant type 1 diabetes, Diabetologia 47 (2004) 1285–1291.
[20] A. Imagawa, T. Hanafusa, J. Miyagawa, Y. Matsuzawa, A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. Osaka IDDM Study Group, N Engl J. Med. 342 (2000) 301–307.
[21] K. Sayama, A. Imagawa, K. Okita, S. Uno, M. Moriwaki, J. Kozawa, H. Iwahashi, K. Yamagata, S. Tamura, Y. Matsuzawa, T. Hanafusa, J. Miyagawa, I. Shimomura, Pancreatic beta and alpha cells are both decreased in patients with fulminant type 1 diabetes: a morphometrical assessment, Diabetologia 48 (2005) 1560–1564.
[22] J.M. Wenzlau, M. Walter, T.J. Gardner, L.M. Frisch, L. Yu, G.S. Eisenbarth, A.G. Ziegler, H.W. Davidson, J.C. Hutton, Kinetics of the post-onset decline in zinc transporter 8 autoantibodies in type 1 diabetic human subjects, J. Clin. Endocrinol. Metab. 95 (2010) 4712–4719.

Endocrine Journal 2010, **57** (11), 947-951

ORIGINAL

Trajectories of anti-islet autoantibodies before development of type 1 diabetes in interferon-treated hepatitis C patients. Case reports and a literature review

Kan Nakamura[1], Eiji Kawasaki[1], Norio Abiru[2], Ozora Jo[2], Keiko Fukushima[2], Tsuyoshi Satoh[2], Genpei Kuriya[2], Masakazu Kobayashi[2], Hironaga Kuwahara[2], Hironori Yamasaki[3], Tatsuya Ide[4] and Katsumi Eguchi[1, 2]

[1]*Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, Nagasaki, Japan*
[2]*First Department of Internal Medicine, Graduate School of Biomedical Science, Nagasaki University, Nagasaki, Japan*
[3]*Center for Health and Community Medicine, Nagasaki University, Nagasaki, Japan*
[4]*Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Fukuoka, Japan*

Abstract. Interferon-alpha (IFN-α) is widely used in the treatment of viral hepatitis, however, it is known that IFN-α therapy may induce type 1 diabetes. We report here on two cases of chronic viral hepatitis C who developed autoimmune type 1 diabetes during Peg-IFN-α plus ribavirin (RBV) therapy. *Case 1*: a 48-year-old male with chronic hepatitis C with chronic thyroiditis. The patient's plasma glucose level was normal and anti-islet autoantibody tests were negative before Peg-IFN-α+RBV therapy. The emergence of glutamic acid decarboxylase 65 autoantibody (GAD65Ab) was observed after five months of treatment. Autoantibodies to insulin and insulinoma-associated antigen-2 (IA-2) also became positive. Eleven months later, thirst and polydipsia occurred with increased fasting plasma glucose level and the patient was diagnosed with type 1A diabetes. Zinc transporter-8 autoantibody (ZnT8Ab) was not detectable at any point. The patient has type 1 diabetes-susceptible HLA-DRB1-DQB1 haplotypes *0405-*0401 and *0901-*0303. *Case 2*: a 65-year-old male with chronic hepatitis C with type 2 diabetes on insulin treatment. GAD65Ab and IA-2Ab were negative before Peg-IFN-α+RBV therapy, however, nine months later, a single appearance of GAD65Ab was observed. After twelve months, his plasma glucose control worsened rapidly, and he was diagnosed with type 1A diabetes. IA-2Ab and ZnT8Ab were negative throughout the clinical course. His HLA-DRB1-DQB1 haplotypes were *0410-*0402 and *1407-*0503. Both cases showed a unique GAD65Ab epitope (amino acids 360-442). These clinical courses suggest that IFN-α therapy provoked acute islet autoimmunity and onset of type 1 diabetes. Therefore, during IFN-α therapy, patients should be closely monitored for the occurrence of type 1 diabetes.

Key words: Type 1 diabetes, Interferon, Glutamic acid decarboxylase 65 autoantibody, HLA

INTERFERON-ALPHA (IFN-α) is widely used in the treatment of viral hepatitis, renal cell carcinoma, chronic myelogenous leukemia, and multiple myeloma for the induction of antiviral proteins and activation of natural killer cells [1]. It is also known that IFN-α therapy may trigger the development of type 1 diabetes, and various patients who developed type 1 diabetes during IFN-α therapy have been reported [2-12]. In this paper, we report on the time course of anti-islet autoantibodies in two cases with type 1 diabetes that developed after Peg-IFN-α+ribavirin (RBV) therapy for chronic hepatitis C.

Case 1

A 48-year-old Japanese male with chronic hepati-

Received Jul. 8, 2010; Accepted Aug. 6, 2010 as K10E-207
Released online in J-STAGE as advance publication Aug. 26, 2010

Correspondence to: Eiji Kawasaki, M.D., Ph.D., Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
E-mail: eijikawa@nagasaki-u.ac.jp

Abbreviations: Ab, autoantibody; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DKA, diabetic ketoacidosis; FPG, fasting plasma glucose; GAD, glutamic acid decarboxylase; HbA_{1c}, hemoglobin A1c; IA-2, insulinoma-associated antigen-2; IAA, insulin autoantibody; IFN, interferon; RBV, ribavirin; ZnT8, zinc transporter-8.

tis C and chronic thyroiditis had received IFN therapy twice in the past. He had no family history of diabetes mellitus. By January 2007, he had undergone IFN therapy a total of three times (Peg-IFN-α2b: Intron A® +RBV: Rebetol®). Before the IFN-α therapy, his hepatitis C virus (HCV) serotype was group 1 and his viral titer was 510 KIU/mL. His plasma glucose level was normal and anti-islet autoantibody tests were negative before treatment. Two months after beginning the treatment, his HCV RNA became negative, and ten months after beginning the treatment, he noticed thirst, polydipsia and polyuria, and was admitted to our hospital. His laboratory data on admission were: fasting plasma glucose (FPG), 154 mg/dL; hemoglobin A1c (HbA_{1C}), 7.6%; aspartate aminotransferase (AST), 27 IU/L; alanine aminotransferase (ALT), 27 IU/L; HCV-RNA, negative; anti-thyroglobulin antibodies, positive (×25600); and anti-thyroid microsomal antibodies, positive (×6400). Fasting serum C-peptide level (3.39 ng/mL) and urinary C-peptide excretion (77 μg/day), were retained. With respect to anti-islet autoantibodies, three of the four analyzed autoantibodies were positive: glutamic acid decarboxylase (GAD)65Ab, 1227 U/mL (normal value <1.4 U/mL); insulinoma-associated antigen-2 (IA-2)Ab, 10.6 U/mL (<0.4 U/mL); and insulin autoantibody (IAA), 1026.1 nU/mL (<125 nU/mL). However, zinc transporter-8 (ZnT8)Ab were negative. The patient's human leukocyte antigen (HLA) haplotypes were DRB1*0405-DQB1*0401 and DRB1*0901-DQB1*0303. Type 1 diabetes was diagnosed and antiviral treatment was withdrawn. Insulin therapy was then initiated.

To determine whether the appearance of anti-islet autoantibodies preceded Peg-IFN-α+RBV therapy, earlier samples were screened for GAD65Ab, IAA, IA-2Ab and ZnT8Ab. Retrospective serology revealed that GAD65Ab was positive after five months, IAA was positive after six months and IA-2Ab was positive after nine months from the initiation of IFN-α therapy (Table 1). In spite of terminating the IFN-α treatment, the patient's C-peptide response to 1 mg i.v. glucagon progressively decreased (Table 2).

Case 2

A 65-year-old Japanese male with chronic hepatitis C and type 2 diabetes had been on insulin treatment for nine years. He had received IFN therapy twice in the past. He had no family history of diabetes mellitus. By January 2007, he had received Peg-IFN-α+RBV therapy a total of three times (Peg-IFN-α2b: Intron A® +RBV: Rebetol®). Eight months after beginning IFN-α therapy, his HCV RNA became negative. Twelve months after beginning treatment, his plasma glucose control worsened rapidly, and he was admitted to our hospital. His laboratory data on admission were: FPG, 91 mg/dL; HbA_{1C}, 8.4%; AST, 31 IU/L; ALT, 28 IU/L; HCV-RNA, negative; thyroid peroxidase antibodies, 113 U/mL (<0.3 U/mL); and thyroid stimulating hormone receptor antibodies, 6.7 IU/mL (1.0 IU/mL). His fasting serum C-peptide level (0.52 ng/mL) and urinary C-peptide excretion (13.2 μg/day) were decreased. GAD65Ab (3520 U/mL) was positive, but IA-2Ab and ZnT8Ab were negative. IAA was not measured because of his insulin treatment before the onset of type 1 diabetes. The patient's HLA haplotypes were DRB1*0410-DQB1*0402 and DRB1*1407-DQB1*0503. Type 1 diabetes was diagnosed and antiviral treatment was withdrawn.

Anti-islet autoantibodies were analyzed using stored sera, revealing that GAD65Ab was positive after nine months from the initiation of IFN-α therapy (Table 1). The patient's C-peptide response to 1 mg i.v. glucagon had been exhausted by the time of the diagnosis of type 1 diabetes (Table 2).

Both patients' GAD65Ab epitope recognition was analyzed using the GAD65/GAD67 chimeric proteins as previously described [13]. Both cases reacted with a unique epitope between amino acids 360-442 of GAD65. Furthermore, the GAD65Ab epitope spread to the C-terminal region (amino acids 443-585) at the onset of type 1 diabetes in Case 2.

Discussion

To the best of our knowledge, this is the first report that describes the time course of anti-islet autoantibodies before the onset of type 1 diabetes induced by IFN therapy in Japanese where the incidence of type 1 diabetes is one of the lowest ethnic groups in the world. In both of our two cases, anti-islet autoantibodies emerged rapidly after the initiation of IFN-α treatment for chronic hepatitis C. After several months from the development of anti-islet autoimmunity, the patients' plasma glucose level was elevated and type 1 diabetes was diagnosed.

Table 3 summarizes the anti-islet autoantibody profile before and after IFN therapy as well as class II

Table 1 Time course of anti-islet autoantibodies in two cases.

	Time (months)	GAD65Ab (U/mL)	IA-2Ab (U/mL)	IAA (nU/mL)	ZnT8Ab (index)	Event
Case 1						
	0	negative	negative	negative	negative	HCV-RNA+
	1	negative	negative	negative	negative	HCV-RNA+
	2	negative	negative	negative	negative	HCV-RNA-
	3	negative	negative	negative	negative	HCV-RNA-
	4	negative	negative	negative	negative	HCV-RNA-
	5	8.5	negative	negative	negative	HCV-RNA-
	7	436	negative	482	negative	HCV-RNA-
	8	942	negative	668.4	negative	HCV-RNA-
	9	1230	4.1	1113.7	negative	HCV-RNA-
	11	1220	8.8	1026.1	negative	T1D onset
	13	1277	10.6	N.D.	N.D.	
Case 2						
	0	negative	negative	N.D.	negative	HCV-RNA+
	1	negative	negative	N.D.	negative	HCV-RNA+
	2	negative	negative	N.D.	negative	HCV-RNA+
	3	negative	negative	N.D.	negative	HCV-RNA+
	4	negative	negative	N.D.	negative	HCV-RNA+
	5	negative	negative	N.D.	negative	HCV-RNA+
	6	negative	negative	N.D.	negative	HCV-RNA+
	9	72.1	negative	N.D.	negative	HCV-RNA+
	10	582	negative	N.D.	negative	HCV-RNA-
	12	3950	negative	N.D.	negative	T1D onset

N.D., not determined. Time = months after the initiation of IFN+RBV therapy. Ab, autoantibody; GAD, glutamic acid decarboxylase; IA-2, insulinoma-associated antigen-2; IAA, insulin autoantibody; ZnT8, zinc transporter-8

Table 2 C-peptide response to 1 mg i.v. glucagon.

	Time (min)	0	1	3	5	10	15
Case 1 (onset)	CPR (ng/mL)	3.39	3.85	5.73	5.03	4.35	3.43
Case 1 (after 7 months)	CPR (ng/mL)	1.52	2.17	3.28	3.42	2.77	2.72
Case 2 (onset)	CPR (ng/mL)	0.08	0.08	0.10	0.09	0.10	0.10

HLA in 17 patients with chronic viral hepatitis who developed type 1 diabetes and who have been reported in the literature and in the present two cases. Anti-islet autoantibody profiles at the onset of diabetes and HLA haplotypes are variable (Table 3). Nine of these 19 patients (47%) were positive for anti-islet autoantibodies before IFN treatment. Seven of the 19 patients (37%), including our two cases, seroconverted during treatment and all of them turned positive for GADAb. The remaining 3 cases (16%) were anti-islet autoantibody negative even after the onset of type 1 diabetes, although data for some autoantibodies such as ZnT8Ab were not available. These results suggest that GADAb may be a good predictive and diagnostic marker for IFN-induced type 1 diabetes, as has been reported in sporadic cases [2-12]. This needs to be verified in the future study using a large number of subjects.

The present Case 1 showed three of the four tested anti-islet autoantibodies, and susceptible HLA-DR-DQ haplotypes, and insulin secretion was retained at di-

Table 3 Anti-islet autoantibody profile before and after IFN therapy and class II HLA in patients with chronic viral hepatitis who developed type 1 diabetes.

Ref.	Age/ sex	IFN	Time (months)	Anti-islet autoantibodies		HLA
				Before IFN	At onset of type 1 diabetes	
2	61/M	α2b	6	GAD (+), ICA (-), IAA (+), IA-2 (-)	GAD (+), ICA (+), IAA (+), IA-2 (-)	DRB1*0401/*1101, DQB1*0502/*0503
3	29/M	α2b	5	GAD (+), ICA (+), IAA (-), IA-2 (-)	GAD (+), ICA (+), IAA (-), IA-2 (-)	DRB1*04/08, DQB1 57 N-Asp/Asp
4	57/M	α2b	4	GAD (-), IAA (-)	GAD (-), ICA (-), IAA (-)	DRB1*0405/*1401, DQB1*0401/*0503
5	41/M	α2b+RBV	3	GAD (-), IAA (-), IA-2 (-)	GAD (-), IAA (-), IA-2 (-)	DRB1*0101/*0401
5	36/F	α2b+RBV	3	GAD (+)	GAD (+)	N.D.
6	29/M	α	8.5	GAD (-), ICA (-), IAA (-), IA-2 (-)	GAD (+), ICA (+), IAA (-), IA-2 (-)	DRB1*0301, DQB1*0201
7	37/M	α2b+RBV	4	GAD (+), ICA (+)	GAD (+), ICA (+)	DR1/3
8	40/F	α2b+RBV	6	GAD (+), ICA (-), IAA(-)	GAD (+)	DR4/7, DQ2/8
8	40/F	α2b+RBV	2	GAD (+), ICA (-), IAA (-)	GAD (+)	N.D.
9	61/M	Peg-α2b+RBV	3	GAD (+), ICA (+), IA-2 (-)	GAD (+), ICA (+), IA-2 (-)	DRB1*04/*14, DQB1*04/*0503
10	42/F	Peg-α2b+RBV	2	GAD (-), ICA (-)	GAD (+), ICA (+)	DR1/4, DQ2/5
11	54/M	Peg-α+RBV	+1	GAD (-), ICA (-), IA-2 (-)	GAD (-), ICA (-), IA-2 (-)	DR3
11	46/M	Peg-α+RBV	3	GAD (+), ICA (-), IA-2 (-)	GAD (+), ICA (-), IA-2 (-)	N.D.
11	25/M	Peg-α+RBV	+1	GAD (-), ICA (-), IA-2 (-)	GAD (+), ICA (+), IA-2 (-)	DR3, DQ2
11	44/F	Peg-α+RBV	6	GAD (-), ICA (-), IA-2 (-)	GAD (+), ICA (-), IA-2 (-)	DR3/4, DQ2
11	46/M	Peg-α+RBV	4	GAD (+), ICA (+), IA-2 (-)	GAD (+), ICA (+), IA-2 (+)	N.D.
12	51/M	Peg-α2b+RBV	6	GAD (-)	GAD (+), IA-2 (-)	N.D.
Case 1	48/M	Peg-α2b+RBV	11	GAD (-), IAA (-), IA-2 (-), ZnT8 (-)	GAD (+), IAA (+), IA-2 (+), ZnT8 (-)	DRB1*0405/*0901, DQB1*0401/*0303
Case 2	65/M	Peg-α2b+RBV	12	GAD (-), IA-2 (-), ZnT8 (-)	GAD (+), IA-2 (-), ZnT8 (-)	DRB1*0410/*1407, DQB1*0402/*0503

N.D., not determined; GAD, GADAb; ICA, islet cell antibody; IAA, insulin autoantibody; IA-2, IA-2Ab; ZnT8, ZnT8Ab.
Time = months after the initiation of IFN therapy; "+1" indicates one month after the end of IFN therapy.

agnosis. In contrast, Case 2 showed only GAD65Ab, has no susceptible HLA-DR-DQ haplotypes, and insulin secretion dried up upon the diagnosis of type 1 diabetes. Thus, it is clear that IFN-α is a common trigger of type 1 diabetes, but clinical courses vary greatly.

It has been reported that IFN-α is overexpressed in the pancreas of patients with type 1 diabetes [14]. Furthermore, in a study using transgenic mice, β cell-specific expression of IFN-α induced by using a monoclonal antibody protected mice from diabetes [15]. In addition, IFN-α is known to induce HLA class I antigen expression, and natural killer cell and T cell activities [16]. However, the underlying mechanisms of IFN-related type 1 diabetes have not yet been clarified. In contrast to the natural history of autoimmune type 1 diabetes, in which the appearance of anti-islet autoantibodies precedes the manifestation of insulin insufficiency by years, established humoral autoimmune markers were seen to have developed up to 3 to 6 months prior to diagnosis in the present cases. It is possible that such a rapid onset is due to acute β cell destruction by IFN; this is supported by the fact that the prediction of type 1 diabetes is difficult in some cases. The nation-wide survey is being executed by Japan Diabetes Society to clarify the clinical and immunogenetic characteristics of IFN-related type 1 diabetes in Japan.

In the present cases, we recognized a unique GAD65Ab epitope. The GAD65Ab epitope located between amino acids 245-360 (E1) is thought to be the marker of acute β cell destruction [13, 17]. However, GAD65Ab E1 was negative and a novel epitope located between amino acids 360-442 was positive in our cases. These results suggest that the underlying mechanism of β cell destruction in patients with IFN-induced type 1 diabetes may be different from that in those with classical type 1A diabetes.

In Case 1, insulin secretion was remarkably decreased at seven months after the discontinuation of IFN-α therapy, suggesting that β cell destruction progressed in spite of the withdrawal of the therapy. In Case 2, insulin secretion had already dried up at the

time of the diagnosis of type 1 diabetes. However, since this patient had been treated with insulin for type 2 diabetes, he did not develop diabetic ketoacidosis (DKA).

In conclusion, the development of type 1 diabetes should be considered a side effect of IFN-α therapy. The onset of disease may be extremely abrupt; therefore, in order to protect patients from the risk of DKA risk, patients receiving IFN-α therapy should be regularly monitored for the presence of anti-islet autoantibodies before and during IFN-α therapy.

Competing Interests

Nothing to declare.

References

1. Sato K, Hida S, Takayanagi H, Yokochi T, Kayagaki N, Takeda K, Yagita H, Okumura K, Tanaka N, Taniguchi T, Ogasawara K (2001) Antiviral response by natural killer cells through TRAIL gene induction by IFN-alpha/beta. *Eur J Immunol* 31: 3138-3146.
2. Fabris P, Betterle C, Floreani A, Greggio NA, de Lazzari F, Naccarato R, Chiaramonte M (1992) Development of type 1 diabetes mellitus during interferon alpha therapy for chronic HCV hepatitis. *Lancet* 340: 548.
3. Fabris P, Betterle C, Greggio NA, Zanchetta R, Bosi E, Biasin MR, de Lalla F (1998) Insulin-dependent diabetes mellitus during alpha-interferon therapy for chronic viral hepatitis. *J Hepatol* 28: 514-517.
4. Uto H, Matsuoka H, Murata M, Okamoto T, Miyata Y, Hori T, Ido A, Hirono S, Hayashi K, Tsubouchi H (2000) A case of chronic hepatitis C developing insulin-dependent diabetes mellitus associated with various autoantibodies during interferon therapy. *Diabetes Res Clin Pract* 49: 101-106.
5. Recasens M, Aguilera E, Ampurdanés S, Sánchez Tapias JM, Simó O, Casamitjana R, Conget I (2001) Abrupt onset of diabetes during interferon-alpha therapy in patients with chronic hepatitis C. *Diabet Med* 18: 764-767.
6. Bosi E, Minelli R, Bazzigaluppi E, Salvi M (2001) Fulminant autoimmune Type 1 diabetes during interferon-alpha therapy: a case of Th1-mediated disease? *Diabet Med* 18: 329-332.
7. Eibl N, Gschwantler M, Ferenci P, Eibl MM, Weiss W, Schernthaner G (2001) Development of insulin-dependent diabetes mellitus in a patient with chronic hepatitis C during therapy with interferon-alpha. *Eur J Gastroenterol Hepatol* 13: 295-298.
8. Mofredj A, Howaizi M, Grasset D, Licht H, Loison S, Devergie B, Demontis R, Cadranel JF (2002) Diabetes mellitus during interferon therapy for chronic viral hepatitis. *Dig Dis Sci* 47: 1649-1654.
9. Cozzolongo R, Betterle C, Fabris P, Paola Albergoni M, Lanzilotta E, Manghisi OG (2006) Onset of type 1 diabetes mellitus during peginterferon alpha-2b plus ribavirin treatment for chronic hepatitis C. *Eur J Gastroenterol Hepatol* 18: 689-692.
10. Tosone G, Borgia G, Gentile I, Cerini R, Conte MC, Orlando R, Piazza M (2007) A case of pegylated interferon alpha-related diabetic ketoacidosis: can this complication be avoided? *Acta Diabetol* 44: 167-169.
11. Schreuder TC, Gelderblom HC, Weegink CJ, Hamann D, Reesink HW, Devries JH, Hoekstra JB, Jansen PL (2008) High incidence of type 1 diabetes mellitus during or shortly after treatment with pegylated interferon alpha for chronic hepatitis C virus infection. *Liver Int* 28: 39-46.
12. Tanaka J, Sugimoto K, Shiraki K, Beppu T, Yoneda K, Fuke H, Yamamoto N, Ito K, Takei Y (2008) Type 1 diabetes mellitus provoked by peginterferon alpha-2b plus ribavirin treatment for chronic hepatitis C. *Intern Med* 47: 747-749.
13. Kawasaki E, Abiru N, Ide A, Sun F, Fukushima T, Takahashi R, Kuwahara H, Fujita N, Kita A, Oshima K, Uotani S, Yamasaki H, Yamaguchi Y, Eguchi K (2003) Epitope analysis of GAD65 autoantibodies in Japanese patients with autoimmune diabetes. *Ann N Y Acad Sci* 1005: 440-448.
14. Huang X, Yuang J, Goddard A, Foulis A, James RF, Lernmark A, Pujol-Borrell R, Rabinovitch A, Somoza N, Stewart TA (1995) Interferon expression in the pancreases of patients with type I diabetes. *Diabetes* 44: 658-664.
15. Stewart TA, Hultgren B, Huang X, Pitts-Meek S, Hully J, MacLachlan NJ (1993) Induction of type I diabetes by interferon-alpha in transgenic mice. *Science* 260: 1942-1946.
16. S Taki. Type I interferons and autoimmunity: lessons from the clinic and from IRF-2-deficient mice (2002) *Cytokine Growth Factor Rev* 13: 379-391.
17. Padoa CJ, Banga JP, Madec AM, Ziegler M, Schlosser M, Ortqvist E, Kockum I, Palmer J, Rolandsson O, Binder KA, Foote J, Luo D, Hampe CS (2003) Recombinant Fabs of human monoclonal antibodies specific to the middle epitope of GAD65 inhibit type 1 diabetes-specific GAD65Abs. *Diabetes* 52: 2689-2695.

Endocrine Journal 2010, **57** (7), 623-628

ORIGINAL

Emergence of anti-islet autoantibodies in Japanese patients with type 1 diabetes

Ichiro Horie[1], Eiji Kawasaki[2], Aya Shimomura[1], Tsuyoshi Satoh[1], Ikuko Ueki[1], Hironaga Kuwahara[1], Takao Ando[1], Norio Abiru[1], Toshiro Usa[1] and Katsumi Eguchi[1, 2]

[1] *First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki 852-8501, Japan*
[2] *Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, Nagasaki 852-8501, Japan*

Abstract. Circulating anti-islet autoantibodies in sera are used as a predictive marker for type 1 diabetes (T1D). We here report two Japanese patients with autoimmune thyroid disease complicated with T1D in whom the time course of anti-islet autoantibodies were observed before the clinical onset of diabetes. Case 1: A woman who had developed Graves' disease at age 25 was diagnosed with type 2 diabetes at age 31; six months later, insulin therapy was started. At age 36 she was diagnosed with T1D due to glutamic acid decarboxylase 65 autoantibodies (GAD65Ab)-positive status and decreased C-peptide levels. With stored sera we retrospectively followed her anti-islet autoantibodies. GAD65Ab, zinc transporter 8 autoantibodies (ZnT8Ab) and insulin autoantibodies (IAA) were found to be positive at age 25. IAA soon turned negative, but GAD65Ab and ZnT8Ab remained positive with high levels. Insulinoma-associated antigen-2 autoantibodies (IA-2Ab) emerged 2 years before the initiation of insulin therapy. She has T1D-susceptible HLA-*DRB1-DQB1* haplotypes, *0405-*0401/*0802-*0302. Case 2: A 49-year-old woman with hypothyroidism due to 19 years' history of atrophic thyroiditis noticed marked thirst, polyuria and weight loss. On admission she was diagnosed as T1D due to GAD65Ab-positive findings and poor C-peptide response to i.v. glucagon. Retrospective serology revealed the emergence of GAD65Ab and IAA just after the clinical onset. IA-2Ab and ZnT8Ab never developed. She has T1D-susceptible and -resistant HLA-*DRB1-DQB1* haplotypes, *0901-*0303/*1502-*0601. The autoantibody profile and the mode of diabetes onset in the two cases were remarkably different. These cases imply that anti-islet autoantibodies do not always precede the onset of T1D.

Key words: Type 1 diabetes, Anti-islet autoantibodies, Prediction, Autoimmune polyglandular syndrome type 3, GAD65

AUTOIMMUNE type 1 diabetes (T1D) is a T cell-mediated, organ-specific immune disease inducing insulin-deficient state as a result of β cell destruction [1]. Since discovery of islet cytoplasmic autoantibodies (ICA) in the sera from patients with T1D in 1974, a number of anti-islet autoantibodies have been identified [2]. Currently, glutamic acid decarboxylase 65 autoantibodies (GAD65Ab), insulin autoantibodies (IAA) and Insulinoma-associated antigen-2 autoantibodies (IA-2Ab) are used for clinical diagnosis and prediction of autoimmune T1D. Recently, zinc transporter 8 (ZnT8) was identified as a novel autoantigen in T1D [3]. It is considered that autoantibodies reactive to the islet-cell proteins are produced following β cell destruction by the T cell, and consequent antigen presentation in the draining lymph nodes, and therefore the antibodies are not causal for development of T1D. Emergence of anti-islet autoantibodies in a patient's sera is considered an indicator of on-going β cell destruction by T cell, and a long prodromal phase before the clinical diabetes onset has been recognized in Caucasian patients. However, presence of anti-islet autoantibodies in Japanese patients with T1D before clinical onset of diabetes has never been reported.

We here report two Japanese patients with autoimmune thyroid disease (AITD) subsequently developed autoimmune T1D (autoimmune polyglandular syndrome type 3; APS 3). Emergence of anti-islet autoantibodies before or at onset of clinical diabetes was confirmed using stored sera from the patients.

Received Mar. 3, 2010; Accepted Apr. 26, 2010 as K10E-068
Released online in J-STAGE as advance publication May 25, 2010

Correspondence to: Eiji Kawasaki, Department of Metabolism/Diabetes and Clinical Nutrition, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. E-mail: eijikawa@nagasaki-u.ac.jp

Methods

We retrospectively examined sera from two Japanese patients with AITD complicated T1D. Both patients had been treated with AITD at our hospital for more than 7 years before clinical onset of T1D. We measured anti-islet autoantibodies in their stored samples.

GAD65Ab (normal range; <1.4 U/mL), IA-2Ab (normal range; <0.4 U/mL), anti-thyroglobulin antibody (TgAb, normal range; <0.3 U/mL) and anti-thyorid peroxidase antibody (TPOAb, normal range; <0.3 U/mL) were measured by the commercially available RIA kit (Cosmic Corporation, Tokyo, Japan). IAA (normal range; <125 nU/mL) and thyroid stimulating antibody (TSAb, normal range; <180 %) were measured by Yamasa Corporation's RIA kit (Chiba, Japan). TSH receptor antibody (TRAb, normal range; <1.0 IU/L) were measured by Yamasa Corporation's RRA kit. ZnT8Ab (normal range; index < 0.007) were measured by radioligand binding assay as previously described [4]. C-peptide (CPR) levels were measured by the commercially available ECLIA kit (Roche Diagnostics K.K., Basel, Switzerland).

Table Laboratory findings on admission.

	Case 1	Case 2	Standard value
Hormonal and immunological analysis			
FPG (mg/dL)	189	374	
F-CPR (ng/mL)	0.33	0.30	
HbA1c (%)	9.9	13.3	4.3-5.8
FT3 (pg/mL)	3.44	2.47	2.37-3.91
FT4 (ng/dL)	1.06	1.58	0.95-1.57
TSH (μU/mL)	5.21	3.590	0.48-5.08
TPOAb (U/mL)	161	230	<0.3
TgAb (U/mL)	1.1	2.9	<0.3
TRAb (IU/mL)	<1.0	3.3	<1.0
TSAb (%)	104	173	<180
GAD65Ab(U/mL)	723.8	10.5	<1.4
IA-2Ab (U/mL)	<0.4	<0.4	<0.4
IAA (nU/mL)	3690.4	340.7	<125.0
ZnT8Ab (Index)	0.075	-0.017	<0.007
Urine			
Ketone	(-)	(+/-)	
U-CPR (μg/day)	7.3	16.6	
HLA typing			
DRB1	*0405/*0802	*0901/*1502	
DQB1	*0401/*0302	*0303/*0601	
Glucagon tolerance test			
0 min CPR (ng/mL)	0.30	0.30	
5 min CPR (ng/mL)	0.67	0.41	
Δ CPR (ng/mL)	0.37	0.11	

Case 1 is taking 10 mg/day methimazole, and case 2 is taking 50 μg/day levothyroxine.

Case reports

Case 1

Case 1 was a 36-year-old woman who had been diagnosed in 1995 at our hospital with Graves' disease, when she was 25 years of age. She was maintained under good control in a euthyroid state by an oral antithyroid drug. One year later, in 1996, she was diagnosed with gestational diabetes mellitus (GDM) and maintained good glycemic control through her pregnancy with diet therapy. After delivery, her glucose tolerance recovered to normal in the 75g oral glucose tolerance test (OGTT) and she maintained good glycemic control. In 2000, during her second pregnancy at age 30, she was diagnosed again with GDM and was prescribed 8 units per day NPH insulin at the maximum. In May, 2001, in the postpartum period, her glucose tolerance recovered to normal in the 75g OGTT. However, several months after delivery her HbA1c levels gradually became elevated (from 5.0 % to 6.3 %) and she was diagnosed with diabetes by the 75g OGTT in September, 2001. When the value reached 7.6 % in February, 2002, insulin therapy was started (6 units per day of premixed insulin). Even under insulin therapy and improved diet and exercise management, her insulin requirement increased to over 30 units per day in 2005. When her HbA1c level worsened over 10% in July, 2006, she was admitted to our hospital.

On physical examination, she was non-obese (height 165.7 cm, weight 59.2 kg and body mass index (BMI) 21.6 kg/m^2), well nourished and in no distress. The significant findings were Graefe's sign, which is a sign of Graves' ophthalmopathy and a diffuse goiter. No diabetic retinopathy or neuropathy was found.

The laboratory findings on admission are summarized in the table. Abnormal findings included hyperglycemia and elevated HbA1c level (9.9 %). The serum level of thyroid hormone was normal, due to her 10 mg/day oral methimazole regimen. The fasting serum C-peptide level was low (0.33 ng/mL) and urinary C-peptide excretion was extraordinarily low, at 7.3 μg/day. Furthermore, the peak serum C-peptide after 1mg i.v. glucagon was 0.67 ng/mL, and she was recognized as being in an insulin-deficient state. Together with the positive results of GAD65Ab (732.8 U/mL) and ZnT8Ab (0.075 index), she was diagnosed as having

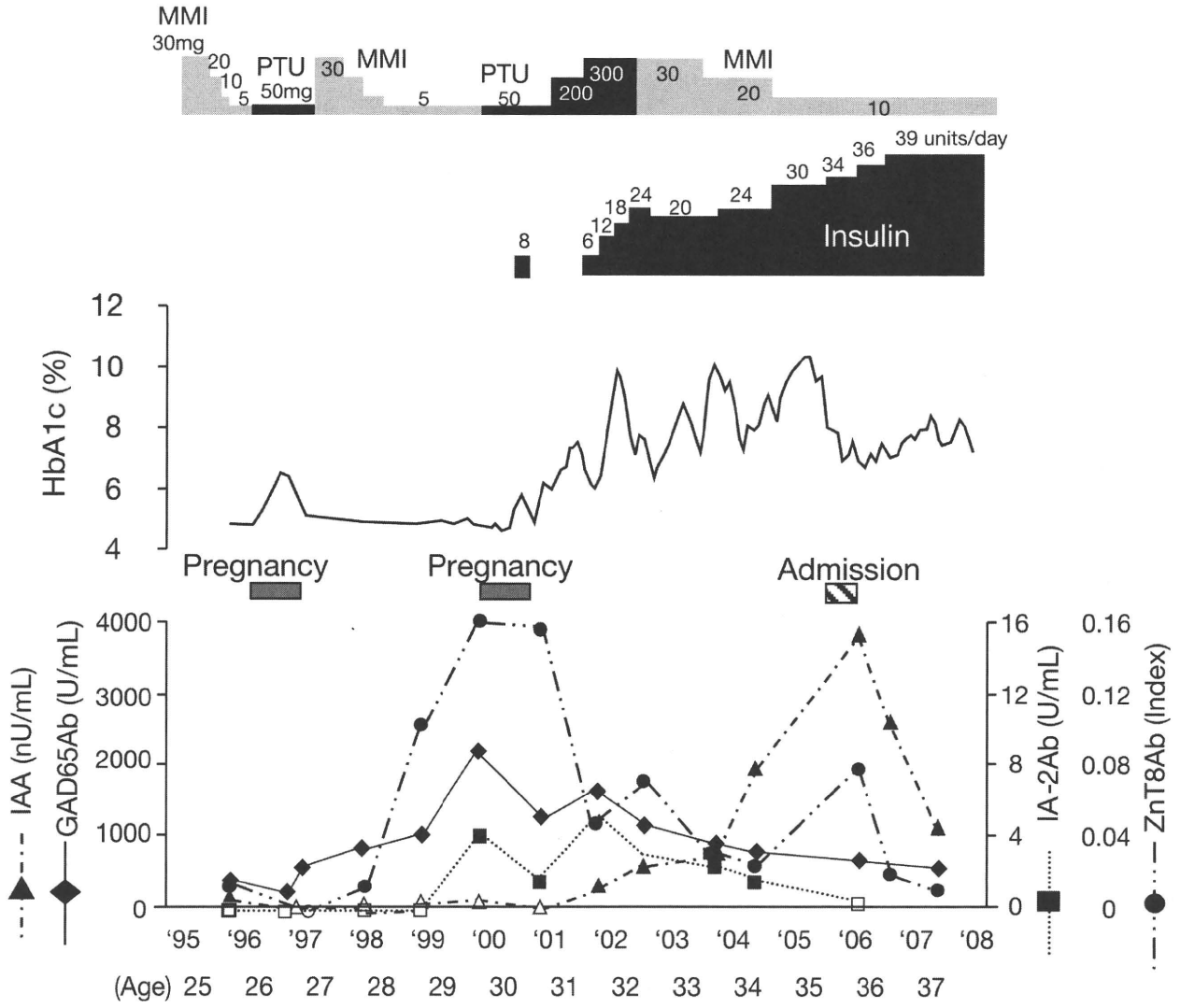

Fig. 1 Clinical course of Case 1. Serum levels of autoantibodies to GAD65Ab, IAA, IA-2Ab and ZnT8Ab are indicated by diamonds, triangles, squares and circles, respectively. The open and closed symbols indicate negative and positive data for the corresponding autoantibody. MMI and PTU indicate methimazole and propylthiouracil.

autoimmune T1D. She had T1D-susceptible HLA-*DRB1-DQB1* haplotypes, *0405-*0401 and *0802-*0302. She had no other autoimmune diseases.

After admission her diabetic control was improved by intensive insulin therapy, comprised of 20 units per day of insulin aspart and 14 units/day of NPH insulin.

As shown in Fig.1, we measured anti-islet autoantibodies using samples obtained over the previous 10 years. GAD65Ab was found to have been positive (386.1 U/mL) in 1996; the GAD65Ab levels were elevated up to 2076.0 U/mL just before she became insulin dependent, and afterwards fell gradually. On the other hand, IA-2Ab became positive (4 U/mL) just before the second pregnancy, 2 years after which she fell into the insulin-dependent state. IAA was also positive (131.0 nU/mL) in 1996, but soon became negative. The fact that it rose again in 2002 after insulin therapy indicates that the therapy induced anti-insulin antibodies. ZnT8Ab was also positive (0.013 index) in 1996, reaching a peak level just before the emergence of IA-2Ab. Her ZnT8Ab reacted with both ZnT8 aa325 variant constructs bearing 325Trp and 325Arg [4].

Case 2

Case 2 was a 49-year-old Japanese woman who was operated on for tetralogy of Fallot at the age of 13 years old, diagnosed with Graves' disease in 1988 at

age 30, and referred to our hospital. She was treated by an oral anti-thyroid drug. As she became hypothyroid without oral anti-thyroid drug one year later, she was diagnosed with atrophic thyroiditis, and we prescribed her levothyroxine (37.5 μg/day; afterwards a dose up to 50 μg/day since 2006). She remained under good control in a euthyroid state.

In November, 2007, at age 49, she noticed severe thirst, polyuria, general fatigue, and weight loss (-11 kg/6 months). On routine follow-up in May, 2008 her plasma glucose level was 751 mg/dL, so she was admitted to our hospital immediately.

On physical examination, she appeared slightly emaciated (height 157 cm, weight 41 kg and BMI 16.6 kg/m^2), with an operation scar on the central sternum and a systolic murmur in 2LSB and 4LSB. Goiter was not palpable. No diabetic retinopathy or neuropathy was found.

The laboratory findings on admission are summarized in the table. The results of complete blood counts and biochemistry were normal except for the diabetes-related data. The HbA1c level was 13.3 %, and the fasting serum C-peptide level was crucially low (0.30 ng/mL) in comparison with the fasting plasma glucose level (374 mg/dL). The urinary C-peptide excretion was also very low (16.6 μg/day). Therefore, it was obvious that impairment of her insulin secretion was severe. Secondary diabetes was ruled out, and she was diagnosed with T1D on the basis of her GAD65Ab-positive (10.5 U/mL) status. She also had no other autoimmune diseases.

After admission her diabetic control was improved by intensive insulin therapy of 36 units per day insulin aspart and 8 units per day insulin glargine. The peak serum C-peptide after 1mg i.v. glucagons at the time of improving from glucose toxicity remained low (0.41 ng/mL). She had both T1D-susceptible and -resistant HLA-*DRB1-DQB1* haplotypes, *0901-*0303 and *1502-*0601.

As shown in Fig. 2, we also analyzed sequential expression of anti-islet autoantibodies and serum glucose levels using the earlier samples. With the sera of before January 8th, 2008, none of anti-islet autoantibodies were detected. GAD65Ab and IAA emerged (1.9 U/mL and 151.1 nU/mL) just after the period (that is April 1st 2008) of appearance of the diabetic symptoms, and IA-2Ab and ZnT8Ab were negative throughout the clinical course. However, serum glucose level was already elevated at 8 months before emergence of anti-islet autoantibodies.

Discussion

It is thought that the anti-islet autoantibodies in patients with T1D do not induce the destruction of the pancreatic islet β cells but are a kind of marker produced by the autoantigens that are leaked and presented in the lymphnodes as a result of β cell destruction.

Although a number of anti-islet autoantibodies have been identified, only 3 markers are currently used for diagnosis and prediction of T1D: GAD65Ab, IA-2Ab and IAA. Recently ZnT8Ab has been identified as the 4th molecularly characterized anti-islet autoantibody [3]. If at least one of these autoantibodies is detected in a patient's serum at the onset of diabetes, that patient is diagnosed with autoimmune T1D, *i.e.* type1A diabetes.

We have previously reported that the prevalences of GAD65Ab, IA-2Ab and IAA were 70 %, 62 %, and 48 %, respectively, in new-onset Japanese patients with T1D, and 89 % were positive for at least one of these autoantibodies [5]. Furthermore, it has been also reported that the level of each autoantibody deteriorates gradually with the duration of T1D and reaches levels below the cut-off. Thus, in general, anti-islet autoantibodies are measured after the clinical onset of T1D, especially in the Japanese population, and there are few reports on the sequential expression of anti-islet autoantibodies before clinical onset of diabetes.

However, in Europe and US, where the prevalence of T1D is much higher than in Japan, such studies in the first-degree relatives of T1D patient have been reported. In the DAISY study in the US, which monitored first-degree relatives of T1D patients who had any anti-islet autoantibodies and whose HLA haplotype was DR3 or DR4, it was reported that GAD65Ab or IAA tended to appear earlier, followed by IA-2Ab, during the pre-diabetic period [6]. Furthermore, it has been also reported that the subjects who had two or more of these autoantibodies had a higher risk for developing T1D compared to those with only 1 autoantibody [7]. Therefore, screening for anti-islet autoantibodies in the prediabetic period is extremely important to predict whether they will develop T1D in the future.

Although the prevalences of anti-islet autoantibodies in new-onset patients with T1D are similar between Japanese [5] and Caucasians [3], it is unknown whether the appearance of autoantibodies before the

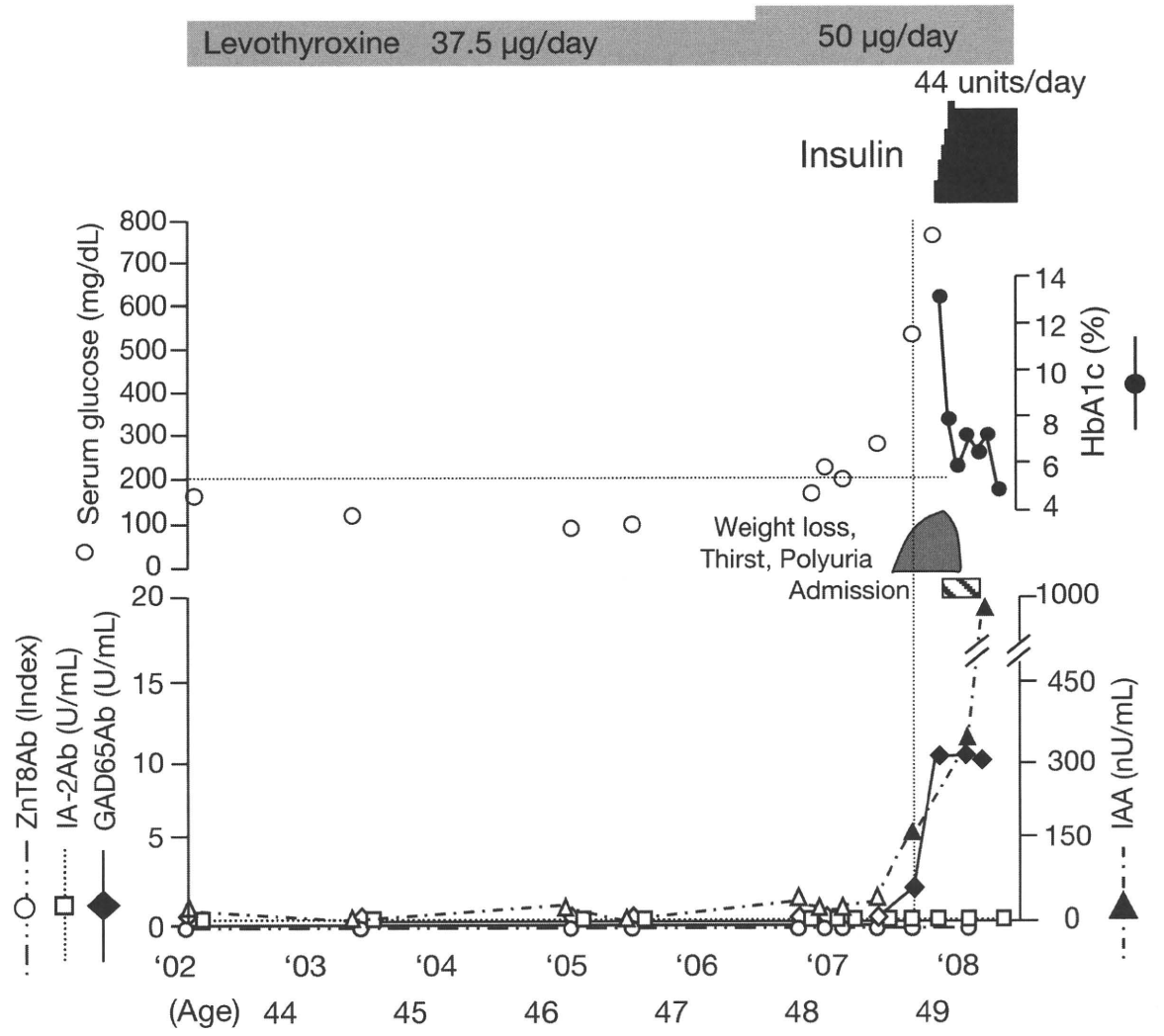

Fig. 2 Clinical course of Case 2. The symbols for each autoantibody are the same as those in Fig. 1. Random serum glucose levels were also analyzed using the earlier samples. Dotted line indicates serum glucose level 200 mg/dL.

clinical onset of T1D in Japanese patients also resembles those in Caucasians. Furthermore, it is difficult to perform such studies in the first-degree relatives of patients with T1D in Japan because the incidence of T1D in Japan is much lower than that in Caucasian populations [8]. During the past 20 years, we have experienced 19 patients with AITD (18 with Graves' disease and one with Hashimoto's thyroiditis) who developed T1D later. Among them we were able to observe the appearance of anti-islet autoantibodies before the clinical onset of T1D only in two patients described here.

In case 1, GAD65Ab and IAA were present more than 7 years before the clinical onset of diabetes, and IA-2Ab emerged about 2 years before the patient became insulin dependent. This time course of autoantibodies is similar to that reported in Caucasians [6]. Of note, ZnT8Ab was also positive 7 years before the clinical onset of diabetes, like GAD65Ab and IAA, and the level of ZnT8Ab increased before the emergence of IA-2Ab.

Case 2 was different from case 1 in terms of the appearance and transition of anti-islet autoantibodies. In this T1D case, GAD65Ab and IAA both emerged just after the clinical onset of diabetes, while IA-2Ab and ZnT8Ab remained negative. According to the reports in Caucasian patients, anti-islet autoantibodies are generally detected in sera of T1D patients more than 10 years before their clinical onset. These facts support the hypothesis by Elliott *et al.* [9], namely, that in the early stage of T1D, residual β cells keep