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ゲノムワイド関連解析による疾患感受性遺伝子・薬剤応答性遺伝子の探索

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1 はじめに

特定の遺伝子の変異が高い確率で発症に結びつく疾患は遺伝病(単一遺伝子疾患)と総称され，5千種類以上もあると推定される一方で各々の有病率は低い．これらとは対照的に，生活習慣病をはじめ日頃よく見聞きするありふれた疾患(common disease)は多因子病あるいは複合病と総称され，様々な環境要因のみならず，多数の遺伝要因も関わっていると考えられる．このような遺伝要因を特定できれば，疾患発症リスクの予測，発症機序の解明，治療法の開発，更には予防にまで役立つことが期待されるが，各々の遺伝子のリスクが高くないためにその特定には多くの困難があった．しかしながら，近年のゲノム科学・技術の進歩により状況は一変している．ここでは我々自身の経験を中心に，統計学的記述は最小限にとどめつつ，疾患感受性遺伝子や薬剤応答性遺伝子をゲノム全域から探索する戦略の現状について解説する．

2 候補遺伝子アプローチとゲノムワイド探索

疾患や治療に関与する遺伝子を含めて，ヒトの様々な特性(表現型)に関わる遺伝子の同定を目指す研究戦略は，候補遺伝子アプローチとゲノム全域へのアプローチに大別される．前者は，推定される病態・特性の分子機序や動物モデルの解析などに基づいて選択された遺伝子について，その変異・多型が患者群に多いかどうか検討する．従来盛んに用いられてきた戦略であり，近年は遺伝子アノテーションやパスウェイ・ネットワーク情報の集積も進んでいることから，ある程度の成功率で疾患関連遺伝子を同定できる戦略である．

一方，後者はゲノム全域をカバーする多数の多型マーカーを用いて統計遺伝学的解析を行うことにより，疾患関連遺伝子を探索する．この戦略の最大の魅力は，我々の知識・情報にはなかった新たな遺伝子の関与が発見される可能性である．多因子疾患に対してよく用いられたゲノム全域探索法が，罹患同胞対(affected sib-pair)を対象とする連鎖解析(linkage study)である．しかし，多数の罹患同胞対試料を収集することは容易ではなく，検出力の低さなどから限られた成果しか得られていなかった．

3 ゲノムワイド関連解析法(GWAS)の進展

2006年以降，疾患関連遺伝子の探索研究は新しい段階を迎えた．これをもたらした最大要因は2つの基盤整備である．まず情報基盤として，dbSNPやHapMap計画*で代表されるように，ヒトゲノム全域にわたる膨大な多様性情報が集積されてきた．次に技術基盤として，数十万種のSNP(single nucleotide polymorphism；単一塩基多型)を数千もの個体について解析できるプラットフォームが市販化された．これらを活用することによって，ゲノムワイド関連解析(genome-wide association study；GWAS)が実用的な戦略となり，2007年には*Nature*，*Science*誌などに立て続けに成果が発表されることとなった．その後の，GWASによる各種疾患の感受性遺伝子の発見ラッシュには目を見張るものがある．

＊ HapMap計画についての用語解説は，414頁参照．

表1　*KCNJ15* の多型は非肥満2型糖尿病と関連する[7]

		試料数	頻度(%)		オッズ比	
			アレル	遺伝子型	アレル	遺伝子型
3つの試料セット総計	健常者	1,700	3.1	6.1	–	–
	全患者	1,568	5.4	10.2	1.76	1.75
	非肥満患者(Ⅰ)[a]	875	5.8	11.1	1.93	1.92
	非肥満患者(Ⅱ)[b]	285	7.6	14.1	2.54	2.51

a)非肥満患者(Ⅰ)群：診断時に肥満でなかった患者(BMI<24).
b)非肥満患者(Ⅱ)群：これまで肥満になったことがない患者(BMI<24).
3つの独立な患者群・健常者群試料セットを合計した値を示す.

GWASの成果が顕著な疾患の1つが2型糖尿病である．2007年に複数の欧米のグループから発表された結果は，いずれも数千人規模のGWASに基づくものであり，合わせて11個以上の感受性遺伝子が同定され，その半数以上が新規に見いだされた遺伝子であった.[1~4] 翌2008年にはその3大グループが共同でメタ解析を行い，更に6個の新規感受性遺伝子を同定している．日本からも2つの独立なグループが，新規遺伝子 *KCNQ1* を報告した.[5,6] 我々もその一方のグループにおいて，大規模SNP解析の一部を担当した.

また我々は最近，GWASではなくゲノムワイド連鎖解析によって検出された候補領域から段階的に絞り込むことによって，新たな感受性遺伝子 *KCNJ15* を同定した.[7] 興味深いことに，見いだされたリスクアレルは2型糖尿病患者全体よりも肥満でない患者とより強い関連を示した．すなわち，BMI(body mass index)が24以上になったことのない患者では，オッズ比が2.5に達した(表1)．肥満でない2型糖尿病患者は，日本をはじめアジア諸国には多いが欧米諸国にはほとんどいない．実際，デンマークの共同研究者によれば，見いだされたリスクアレルの頻度は1%以下であった．興味深いことに，この遺伝子はインスリン産生の制御に関わる遺伝子と考えられ，特に高血糖時のインスリン産生誘導に抑制的な機能を持つと推定された(図1)．

代表的な過眠症であるナルコレプシーの発症にも，ストレスなどの環境要因に加えて複数の遺伝要因が関わると考えられる．従来，遺伝要因として確定しているのは *HLA-DQB1* 遺伝子のみであったため，我々がGWASを用いて探索した結果，22番染色体上の *CPT1B* 及び *CHKB* 遺伝子近傍の多型がナルコレプシーに関連することを見いだした(図2).[8] このリスクアレルは両遺伝子の低発現レベルと関連した(図3)．さらに，スタンフォード大を中心とする国際共同研究グループのGWASによって，新たな感受性遺伝子 *TCRA*(T細胞リセプターα)を見いだすことができた(表2).[9] *TCRA* と上述した *HLA* とは，多様な抗原に対する免疫応答性を制御する主役であることが知られている．これらの成果から，我々はナルコレプシー発症には少なくとも2つの機序，すなわちオレキシン(ヒポクレチン)産生細胞に対する自己免疫，及び脂肪酸β酸化あるいはコリン代謝系の異常が関与していると推定している.

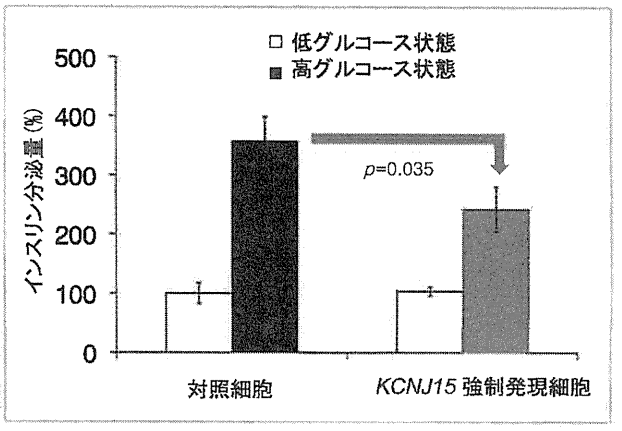

図1　インスリン分泌細胞に *KCNJ15* を強制発現すると応答性インスリン分泌量が減少する[7]

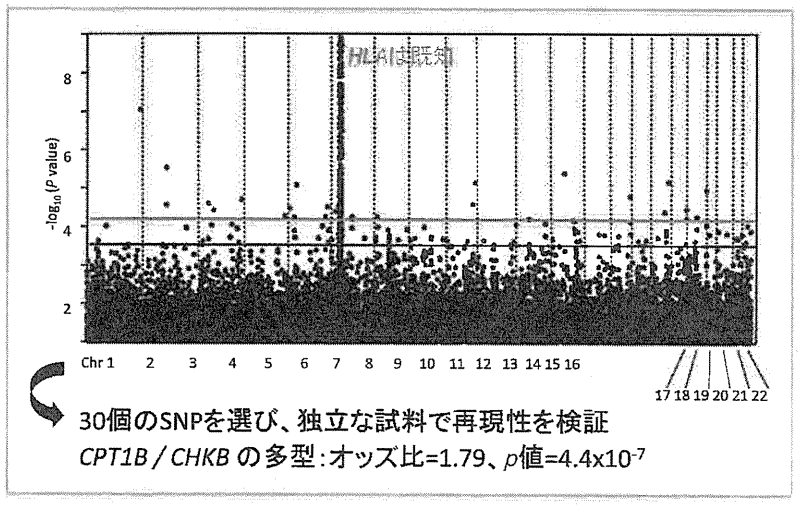

図2　GWASで検出されたナルコレプシーの感受性座位[8]

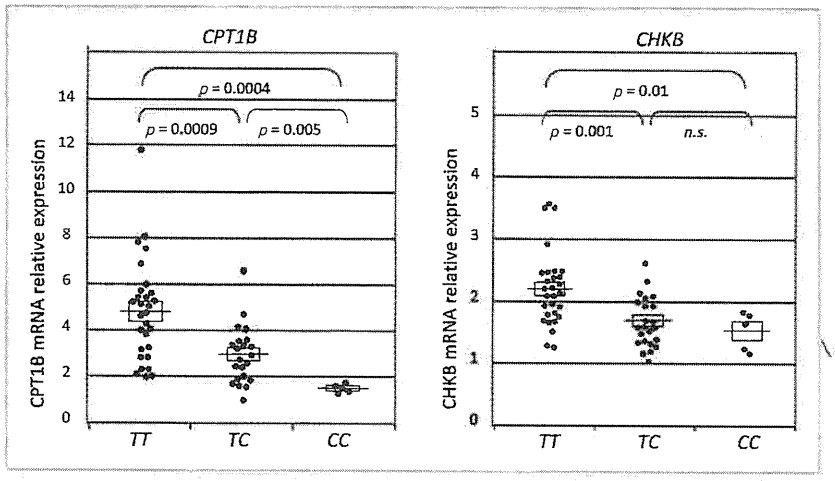

図3　感受性アレルは *CPT1B* 及び *CHKB* の低発現レベルと関連する[8]
末梢血白血球中のmRNA発現レベルをリアルタイムPCR法で定量した．

表2　*TCRA* の多型とナルコレプシーの関連[9]

集団	rs 12587781 C	rs 1154155 C	rs 1263646 G
ヨーロッパ系集団			
患者中頻度	0.22	0.22	0.24
健常者中頻度	0.14	0.14	0.16
p 値	3.58×10^{-5}	3.67×10^{-5}	2.19×10^{-4}
オッズ比	1.79	1.80	1.65
日本人及び韓国人			
患者中頻度	0.68	0.57	0.51
健常者中頻度	0.61	0.47	0.45
p 値	8.70×10^{-4}	2.30×10^{-7}	1.73×10^{-3}
オッズ比	1.34	1.54	1.30

4　GWASによる治療・薬剤応答性遺伝子の探索

ウイルス性肝炎に関する多施設共同研究においても，我々はGWASを担当している．最近，C型肝炎のペグインターフェロンα・リバビリン併用療法の無効例と極めて強く関連する遺伝子として，全く予想されていなかった19番染色体上の *IL28B* 遺伝子を見いだした．[10] すなわちGWAS段階で既に10^{-12}レベルのp値を示し，ゲノムワイド有意水準をクリアした(図4)．再現性検討段階を合わせるとp値は10^{-27}～10^{-32}となり，本治療法の無効性に関

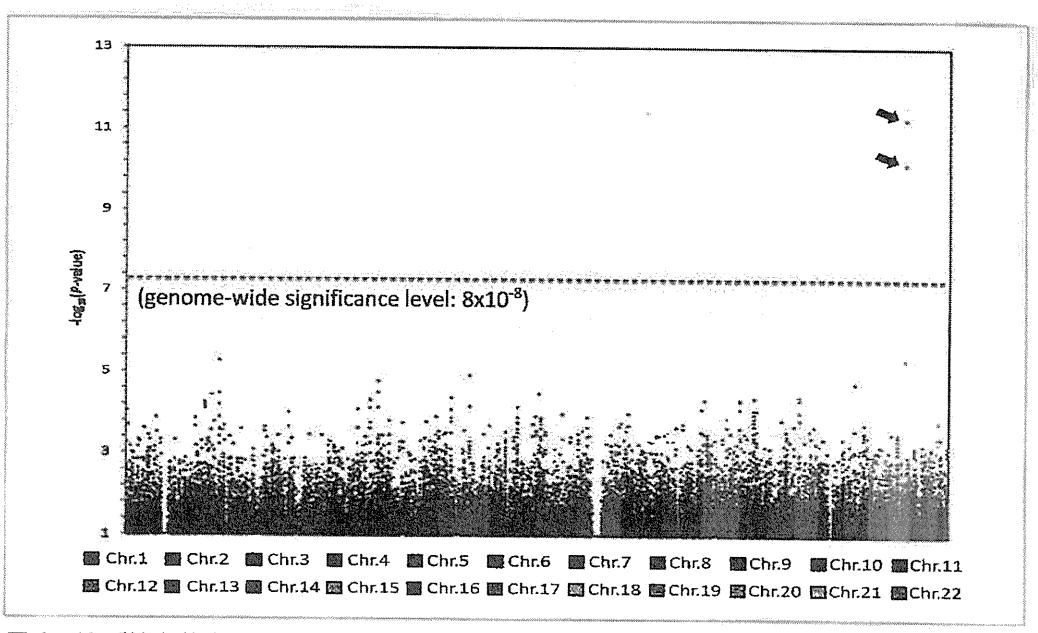

図4　19番染色体上の2個のSNPがGWAS段階でゲノムワイド有意水準の関連を示した[10]

してマイナーアレル陽性患者群のオッズ比は17～30に達した(図5)．またリスクアレルを持つ患者群では，*IL-28* の発現レベルが有意に低いことも分かった．従来，ウイルスの型や量が主に関わると考えられてきたインターフェロン療法への応答性が，実はほぼヒトの遺伝要因で決定されていることの発見は大きなインパクトを持つ．さらに興味深いことに，*IL28B* はインターフェロンλファミリーの一員であり，治療に用いられるインターフェロンαと類似するレセプター及び細胞内シグナル伝達系を介して，ウイルス感染に対する防御作用を発揮する．したがって，治療効果の見込めない患者の予測のみならず，新たな治療薬の開発への貢献が期待される．

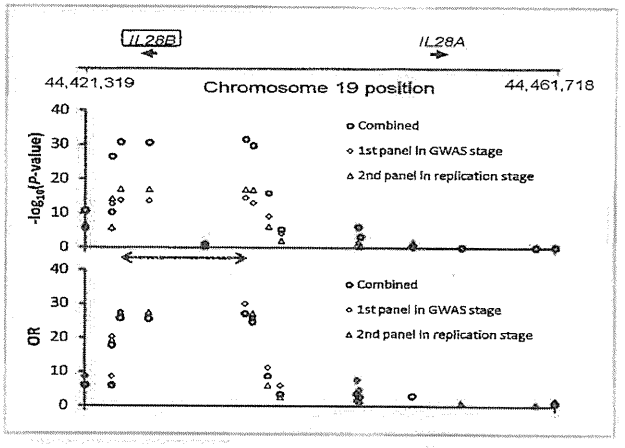

図5　C型肝炎のPEG-IFN-α/RBV治療応答性が *IL28B* 遺伝子領域にマップされた[10]

このほか，各種の薬剤に対する反応性に関わる遺伝子が報告されており，GWASによって初めて同定された遺伝子も増えている．また，薬剤過敏症に関わる遺伝子の探索においても，GWASが用いられる研究が多くなっている．一般に薬剤応答性遺伝子は，疾患感受性遺伝子に比較してオッズ比が大きい傾向にあるため，比較的少数の試料でも遺伝子を特定できる可能性が高いことから，今後ますますの成果が期待される．

5　疾患関連遺伝子に見られる集団差

次に，先に述べた2型糖尿病の感受性遺伝子に見られた集団差を取り上げたい．表3は，ヨーロッパ系集団で見いだされた代表的な2型糖尿病感受性遺伝子 *TCF7L2* と，日本人で見いだされた *KCNQ1* について，両集団におけるアレル頻度，オッズ比，*p* 値を比較したものである．*TCF7L2* は，ヨーロッパ系集団では 10^{-48} と明確な関連を示すことが分かる．一方，日本人では *p* 値が 10^{-4} レベルにとどまるが，その最大の理由はマイナーアレルの頻度の違

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．

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．

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．

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

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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.

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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.

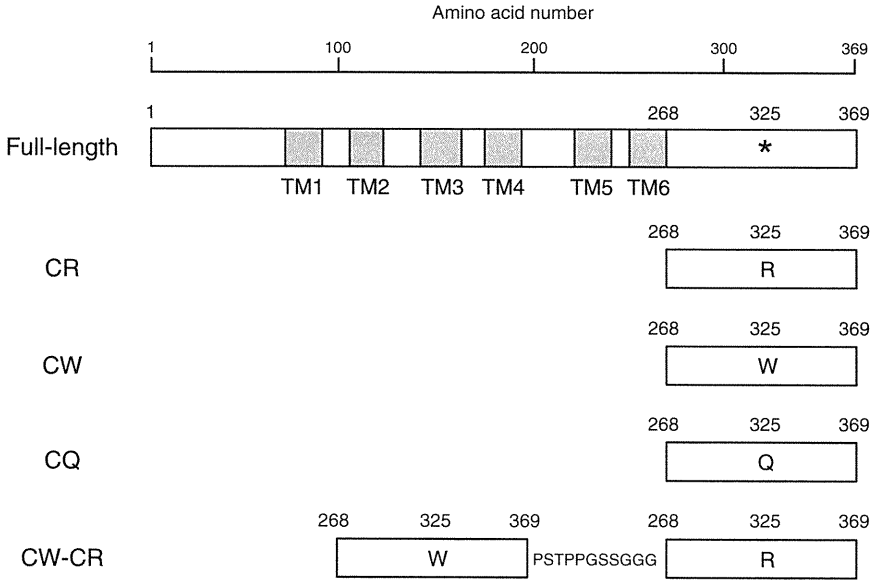

Figure 1 Schematic representation of full-length human ZnT8 and the amino acid boundaries of four constructs used in this study. Numbers correspond to the amino acid residues of the ZnT8 published sequence [4,6]. A hinge sequence of CW-CR construct is derived from the human IgG heavy chain. TM, transmembrane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

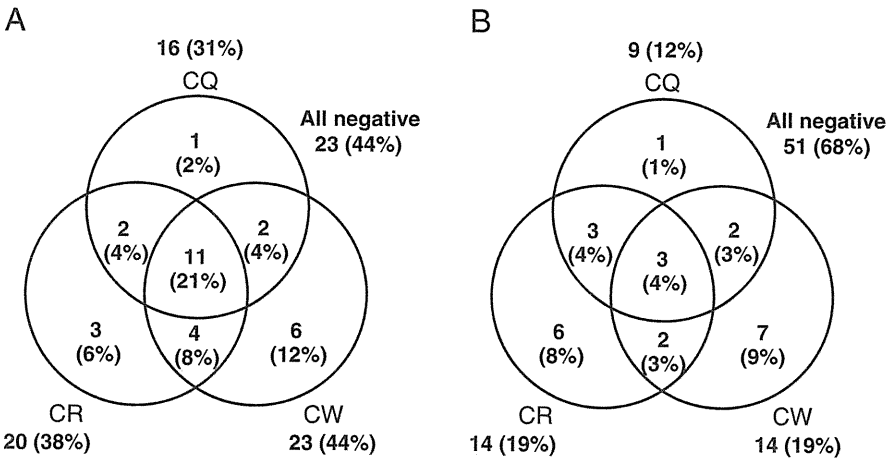

Figure 2 Humoral autoreactivity to 325Trp (CW), 325Arg (CR), and 325Gln (CQ) constructs in patients with childhood-onset (A) and adult-onset type 1 diabetes (B). Venn diagram illustrates the overlap of autoantibody detection with each of the polymorphic construct in 52 childhood-onset and 75 adult-onset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

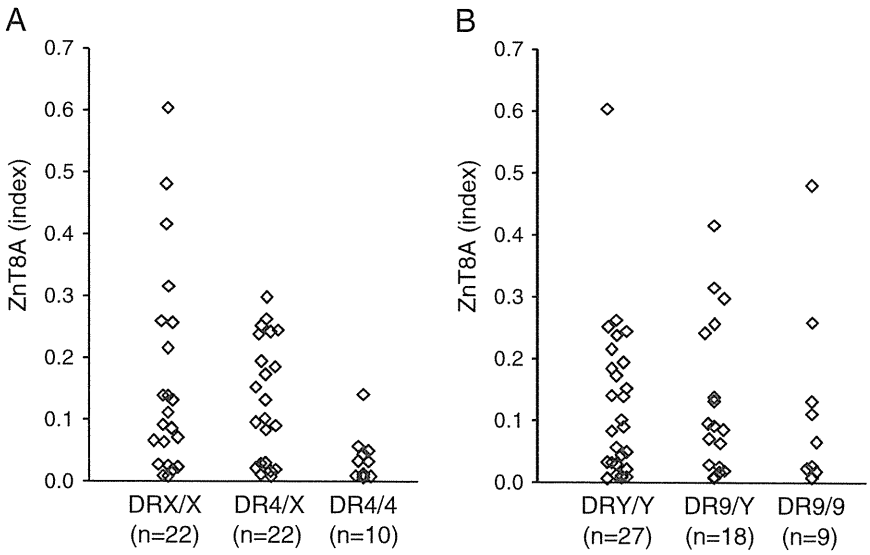

Figure 3 Comparisons of the level of ZnT8A with the copy number of the HLA-*DR4* (A) and -*DR9* (B). "X", non*DR4* allele; "Y", non*DR9* allele (B). The levels were compared among the ZnT8A-positive individuals. The mean (±SD) index of ZnT8A is 0.041 (±0.040) for HLA-*DR4* homozygotes, 0.132 (±0.097) 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).

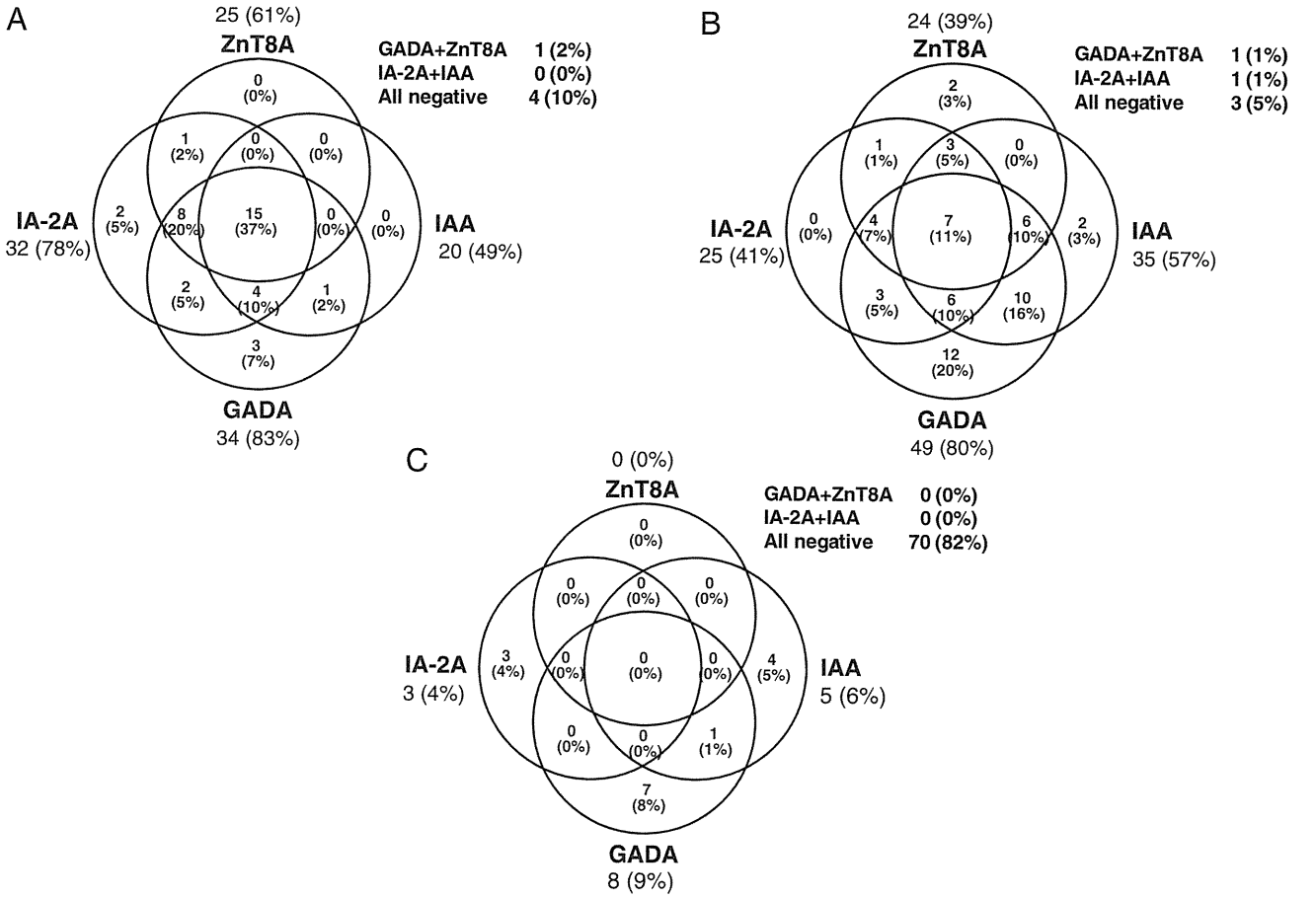

Figure 4 Combinatorial analysis of autoantibodies to ZnT8, GAD65, insulin, and IA-2. A, Childhood-onset type 1 diabetes (n=41); B, adult-onset type 1 diabetes (n=61); C, fulminant type 1 diabetes (n=85). Patients' sera obtained within two weeks after the initiation of insulin treatment were used.

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

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

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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-

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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.

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