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ORIGINAL

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

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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.

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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

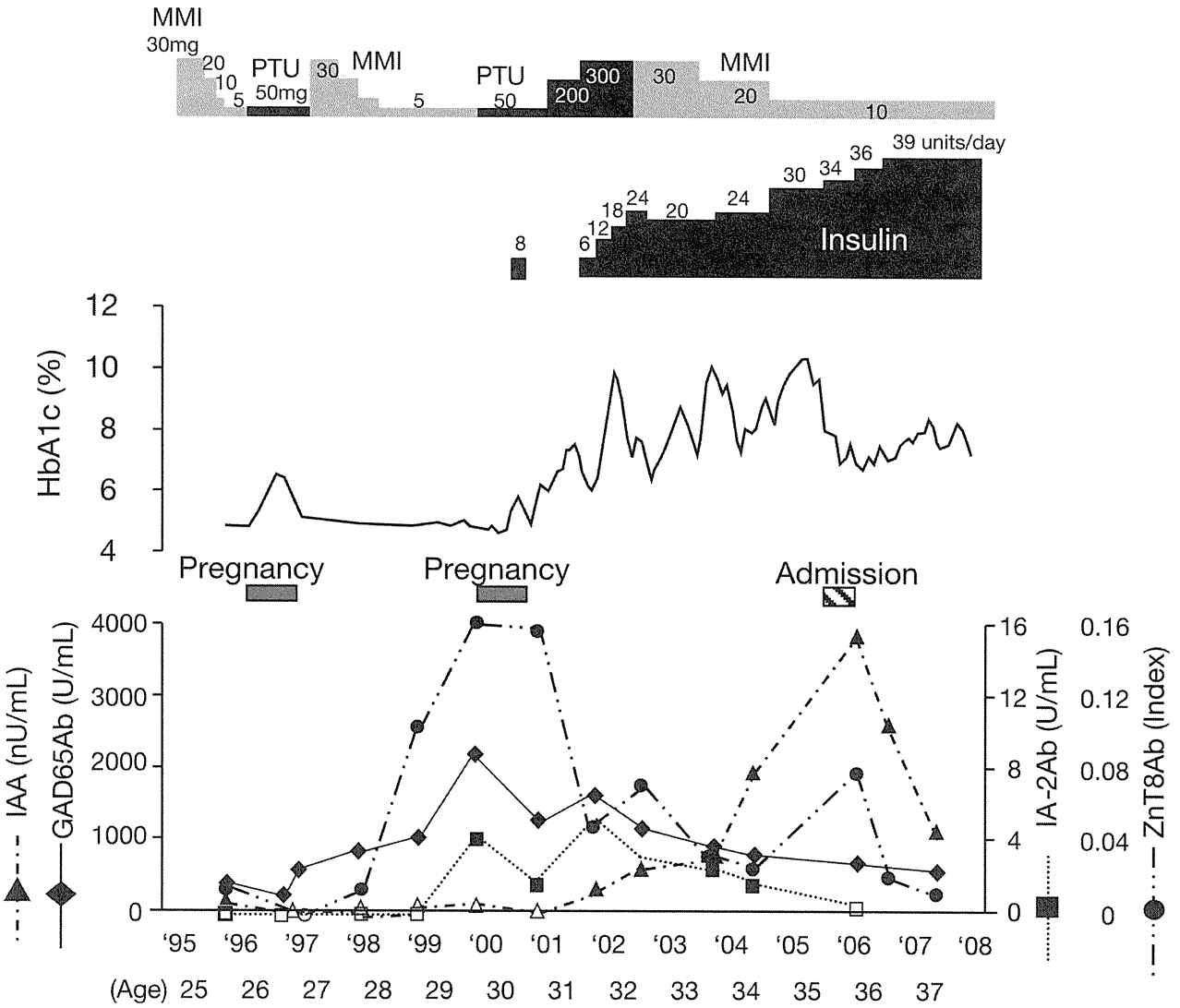

Fig. 1 Clinical course of Case 1. Serum levels of autoantibodies to GAD65Ab, IAA, IA-2Ab and ZnT8Ab are indicated by diamonds, triangles, squares and circles, respectively. The open and closed symbols indicate negative and positive data for the corresponding autoantibody. MMI and PTU indicate methimazole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

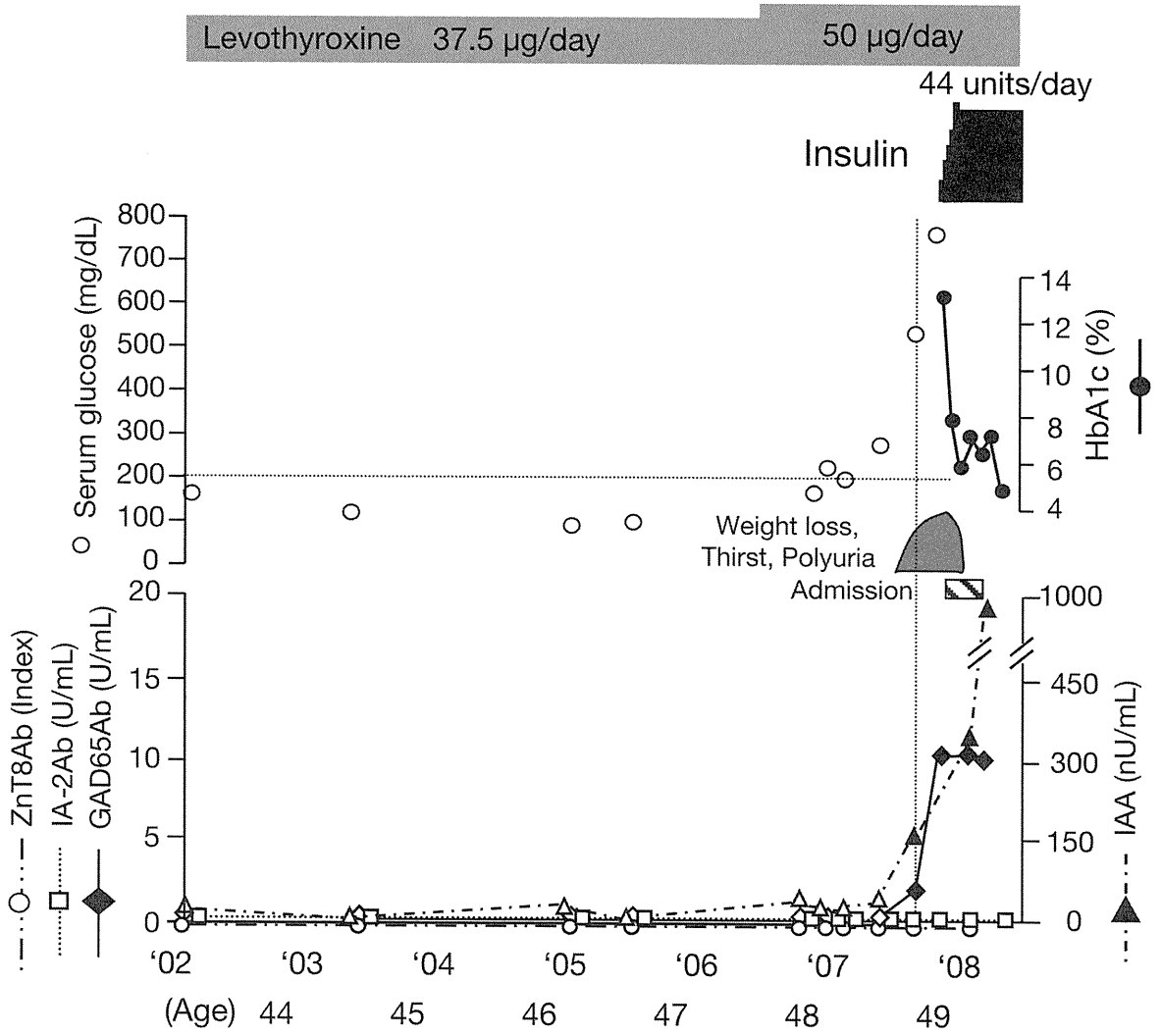

Fig. 2 Clinical course of Case 2. The symbols for each autoantibody are the same as those in Fig. 1. Random serum glucose levels were also analyzed using the earlier samples. Dotted line indicates serum glucose level 200 mg/dL.

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

glucose tolerance normal even if the autoimmune attack to pancreatic islet β cells begins, and overt diabetes develops when β cell function cannot maintain the normal plasma glucose level after the progression of β cell destruction. Moreover, the *in vivo* study by Sreenan *et al.* [10] with non-obese diabetic (NOD) mice, an animal model of human T1D, supports this hypothesis. They recognized that the plasma glucose levels of NOD mice rose after the destruction of more than 70 % of their islet cells by infiltrating lymphocytes.

The time course of anti-islet autoantibodies in case 1 supports these reports. It is possible that most of the pancreatic islet β cells in this case were destroyed in a short period. However, in case 2, it may have been difficult to predict her clinical onset of T1D even if she had done careful follow-up by the attending physician because anti-islet autoantibodies emerged just after the clinical onset of diabetes. Much lower titer of anti-islet autoantibodies in case 2 compared to case 1 may reflect her mode of diabetes onset. Although she has one of the resistant HLA haplotypes in the Japanese population, its contribution to her time course of anti-islet autoantibodies is unknown.

These results imply that the mode of development of anti-islet autoantibodies before clinical onset of T1D is variable in Japanese patients, and that the onset of T1D is not always preceded by the presence anti-islet autoantibodies.

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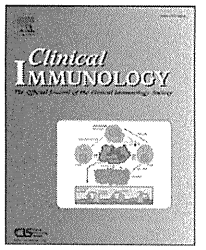

Administration of a determinant of preproinsulin can induce regulatory T cells and suppress anti-islet autoimmunity in NOD mice

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Leader peptide;
NOD;
Regulatory T cell

Abstract Antigen-specific immunotherapy is expected to be an ideal strategy for treating type 1 diabetes (T1D). We investigated the therapeutic efficacy of a peptide in the leader sequence of preproinsulin, which was selected because of its binding affinity to the MHC I-A^{g7} molecule. Preproinsulin-1 L7–24 peptide (L7–24) emulsified in Freund's incomplete adjuvant was administered subcutaneously to NOD mice. Administration of L7–24 increased the proportion of regulatory T cells in the spleen. Splenocytes of NOD mice immunized with this peptide secreted IL-4 and IL-10 in response to L7–24. This peptide also significantly prevented the development of diabetes and cured some newly diabetic NOD mice without recurrence. L7–24 peptide, which has a high affinity for pockets of I-A^{g7}, induced regulatory T cells and showed therapeutic effects. This peptide may provide a new approach for developing antigen-specific immunotherapy for autoimmune diabetes.

Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease characterized by progressive loss of β cells [1]. Autoreactive T cells that react with β cell antigen play a major role in β cell destruction. Several treatments have been attempted to suppress these autoreactive T cells. Cyclosporin slows the autoimmune process and improves residual β cell function, although the positive effect is transient and the drug cannot be applied in clinical practice because of adverse side effects [2,3]. So far, treatment with anti-CD3 monoclonal antibodies seems to be the most effective [4,5], but the preventive effect is limited and this drug may cause general immune suppression.

Administration of autoantigen is expected to become a therapy to establish self-tolerance without severe adverse effects. A recent study demonstrated that subcutaneous injection of glutamic acid decarboxylase (GAD), an important autoantigen in the development of T1D, contributes to the preservation of residual insulin secretion in patients

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Table 1 Sequence of L7–24, B9–23, and HEL10–23.

L7–24	FLPLLA**LLA**LW**E**PKPTQA
B9–23	SHLV**E**ALYLVCG**E**RG
HEL10–23	AAMKRHGL**D**NYRGY

Amino acids that have a high affinity for pockets of MHC I-A^{g7} are shown with thick characters.

with alrecent-onset T1D, although it cannot halt the disease process [6].

Several autoantigens involved in the development of T1D have been identified, and there is increasing evidence that insulin is the primary autoantigen among them [7–9]. However, randomized controlled clinical trials of insulin have shown that it cannot delay or prevent the development of T1D [10,11], possibly because of the limited antigen dose, the timing of administration, or a critical epitope in the C-peptide or leader sequence.

In our study, we focused on a determinant of a signal peptide as a new candidate for immunotherapy. Mice have two preproinsulin genes, and the preproinsulin-1 L7–24 peptide (L7–24) was selected as the therapeutic peptide because these amino acids match the unique pockets of MHC I-A^{g7} of NOD mice [12] and peptidase cleavage sites. The peptide was administered to NOD mice to evaluate its protective effects on the development of diabetes.

Materials and methods

Mice

NOD/Shi/Kbe mice were maintained in the Institute for Experimental Animals, Kobe University School of Medicine. Insulin 2 knockout NOD mice were a gift from Dr. George Eisenbarth at the Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center. All experiments were done using female mice. All animals were housed in specific pathogen-free facilities and handled under the Guidelines for Animal Experimentation of Kobe University School of Medicine.

Peptides

HPLC-purified (>95%) peptides were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Amino acid sequences of the synthesized peptide used in our study were as

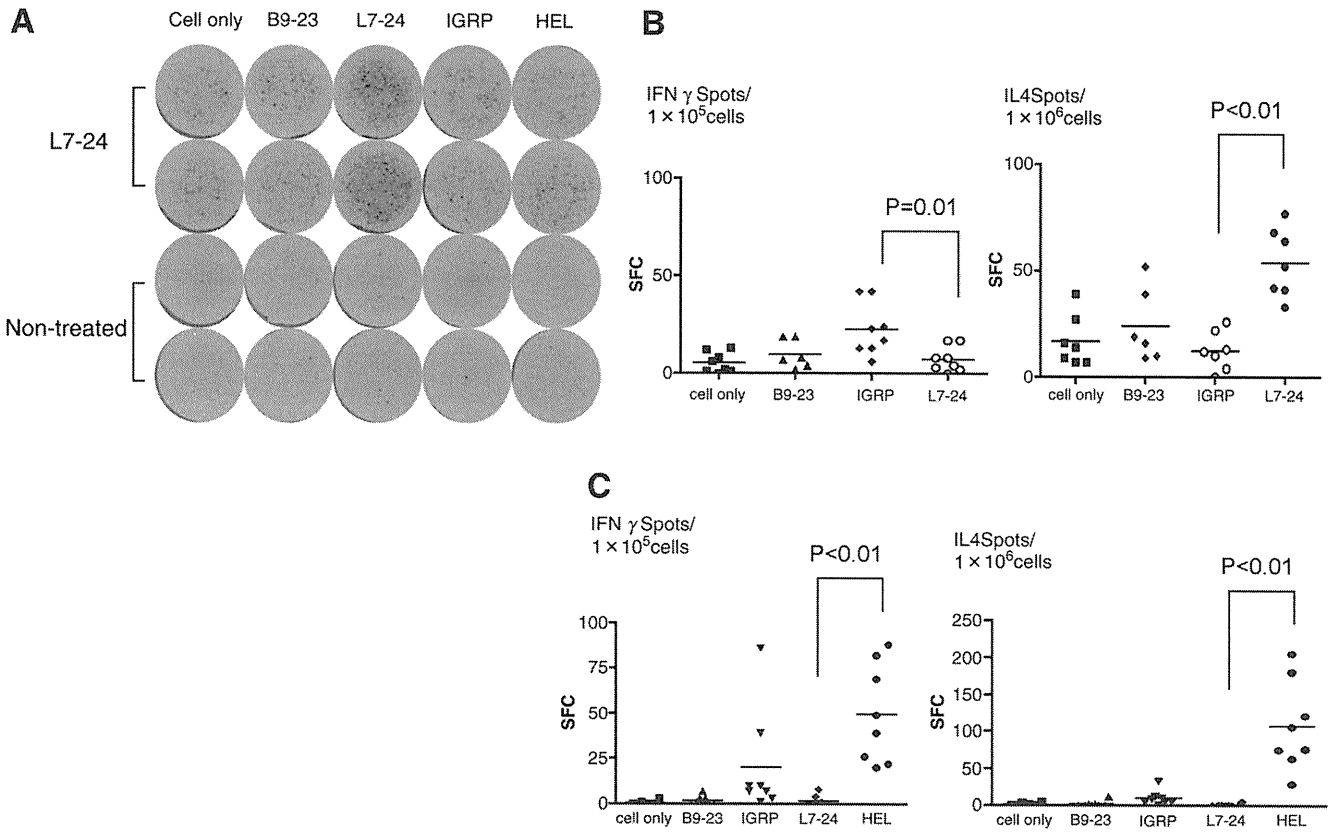

Figure 1 IFN-γ and IL-4 ELISPOT assay for splenocytes from L7–24 peptide-immunized mice (A and B) or HEL10–23 peptide-immunized mice (C). L7–24 or HEL10–23 peptide was injected subcutaneously (s.c.) into NOD mice twice weekly. Seven days after the last immunization, spleen cells were cultured with antigenic peptides (B9–23, IGRP206–214, and L7–24) in duplicates and analysed using an ELISPOT assay. A representative image of an IL-4 ELISPOT assay for splenocytes from L7–24 peptide-immunized mice is shown in (A). The antigen-stimulated spots were quantified as the mean number of IFN-γ spots/1 × 10^5 splenocytes or IL-4 spots/1 × 10^6 splenocytes (n=7), and individual IFN-γ or IL-4 spots of splenocytes from L7–24 peptide-immunized mice (B) or HEL10–23 peptide-immunized mice (C) are depicted as separate dots. Each bar indicates the mean of the spots. Spleen cells of L7–24 peptide-immunized NOD mice showed vigorous secretion of IL-4 and the lack of Th1 reaction, whereas spleen cells of HEL10–23 peptide-immunized mice show both Th1 and Th2 cells.

follows: preproinsulin-1 L7–24 (FLPLLALLALWEPKPTQA), insulin-2 B9–23 (SHLVEALYLVCGERG), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) 206–214 (VYLKTNVFL), and hen egg lysozyme (HEL) 10–23 (AAMKRHGLDNYRGY).

Peptide administration

Each peptide (100 μg/mouse) in PBS was emulsified with Freund's incomplete adjuvant (IFA, Sigma-Aldrich Japan) and administered subcutaneously (s.c.) to posterior cervical region of the mice. Two subcutaneous administrations were conducted 1 week apart in all immunization. The blood glucose concentration was measured weekly using a FreeStyle® kit (Nipro, Osaka, Japan), and the mice were considered to be diabetic after two consecutive blood glucose (BG) values >300 mg/dL.

Islet-infiltrating cell isolation

Islets were isolated as described previously [13]. Briefly, collagenase (Wako Pure Chemical Industries, Osaka, Japan) was injected through the bile duct into the pancreas. After incubation at 37 °C for 10 to 16 min, islets were isolated by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich Japan) and filtered through a cell strainer (BD Falcon, Franklin Lakes, NJ) to remove islets.

ELISPOT

A murine ELISPOT assay was performed using a modification of the human ELISPOT assay described previously [14,15]. In brief, splenocytes (1×10^5 for IFN-γ, 1×10^6 for IL-4) or islet-infiltrating cells (1×10^5) were cultured with antigenic peptides (50 μg/ml) in 96-well microtiter plates (Millititer, Millipore, Bedford, MA) in 200 μl of RPMI 1640 with 5% FCS for 48 h for IFN-γ and 70 h for IL-4. The ELISPOT assay was performed in duplicate well according to the manufacturer's instructions (Mabtech AB, Stockholm, Sweden). Spots were analyzed by the readers blinded to the nature of the sample with an ImmunoSpot Analyzer® (Cellular Technology, Cleveland, OH), and the spot size cutoff was determined at 0.0052 mm^2.

ELISA

The supernatant from splenocytes cultured with antigenic peptides was used for the ELISA. IL-10 concentration was measured using a Biotrak Easy ELISA® (GE Healthcare, Buckinghamshire, UK) and TGF-β concentration was measured using a Quantikine® immunoassay kit (R&D Systems,

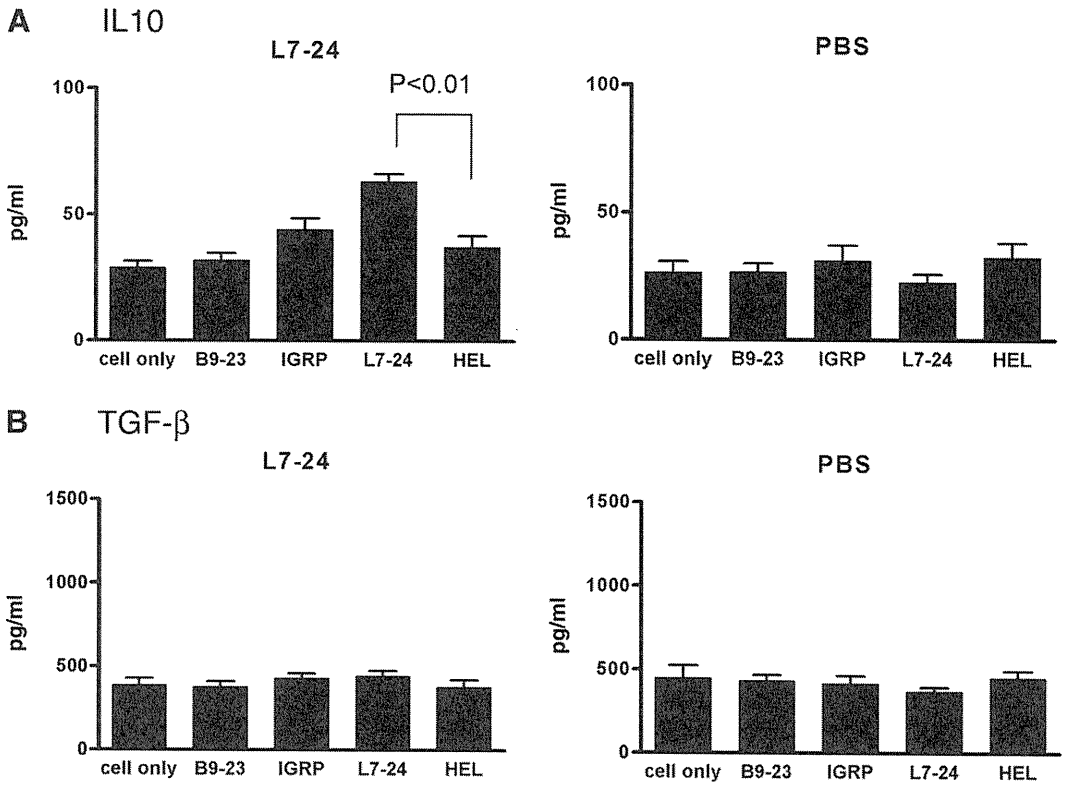

Figure 2 IL-10 and TGF-β ELISA assay for splenocytes from L7–24 peptide-immunized mice or unimmunized mice. Splenocytes (1×10^6) from immunized mice ($n=8$) and control mice ($n=8$) were cultured with antigenic peptides. The concentrations of IL-10 (A) and TGF-β (B) in the supernatant were measured by ELISA. Control mice were immunized with PBS emulsified in IFA. A significantly higher concentration of IL-10 was observed in the supernatant of splenocytes from mice treated with L7–24.

Minneapolis, MN) according to the manufacturers' instructions.

Flow cytometry

Spleen cells or islet-infiltrating cells were stained with anti-CD4 and anti-CD25 monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA) for 30 min at 4 °C. Cells were permeabilized and stained using a Foxp3 staining buffer set according to the manufacturer's instructions (eBioscience, San Diego, CA).

Islet histology

The pancreas was fixed in 10% formalin solution, embedded in paraffin, and sectioned at a thickness of 5 µm. The sections were stained with hematoxylin and eosin. Immunostaining for insulin and glucagon was performed using guinea pig antibodies against insulin and rabbit antibodies against glucagon (Dako Japan, Kyoto, Japan). The immune complexes were detected with secondary antibodies conjugated with Cy3 and fluorescein isothiocyanate, respectively (Jackson ImmunoResearch Laboratories, West Grove, PA).

Statistical analysis

Student's *t* test was used to compare the means. A log-rank test was used to compare the incidence of diabetes between groups. GraphPad Prism 4® for Windows (GraphPad Software, San Diego, CA) was used for these analyses.

Results

Determinants of preproinsulin with high affinity to I-A^{g7}

Sequences of preproinsulin 1 and 2 were investigated to find a new immunological determinant with therapeutic effect against autoimmune diabetes. Four amino acids of the peptide are keys to determine the affinity to the binding pockets of MHC I-A^{g7} [12]. We found that the leader peptide of preproinsulin 1 contained three of these amino acids in their sequence (Table 1): 13 (leucine) matches to P4, 15 (alanine) to P6, and 18 (glutamic acid) to P9 of I-A^{g7} binding sites.

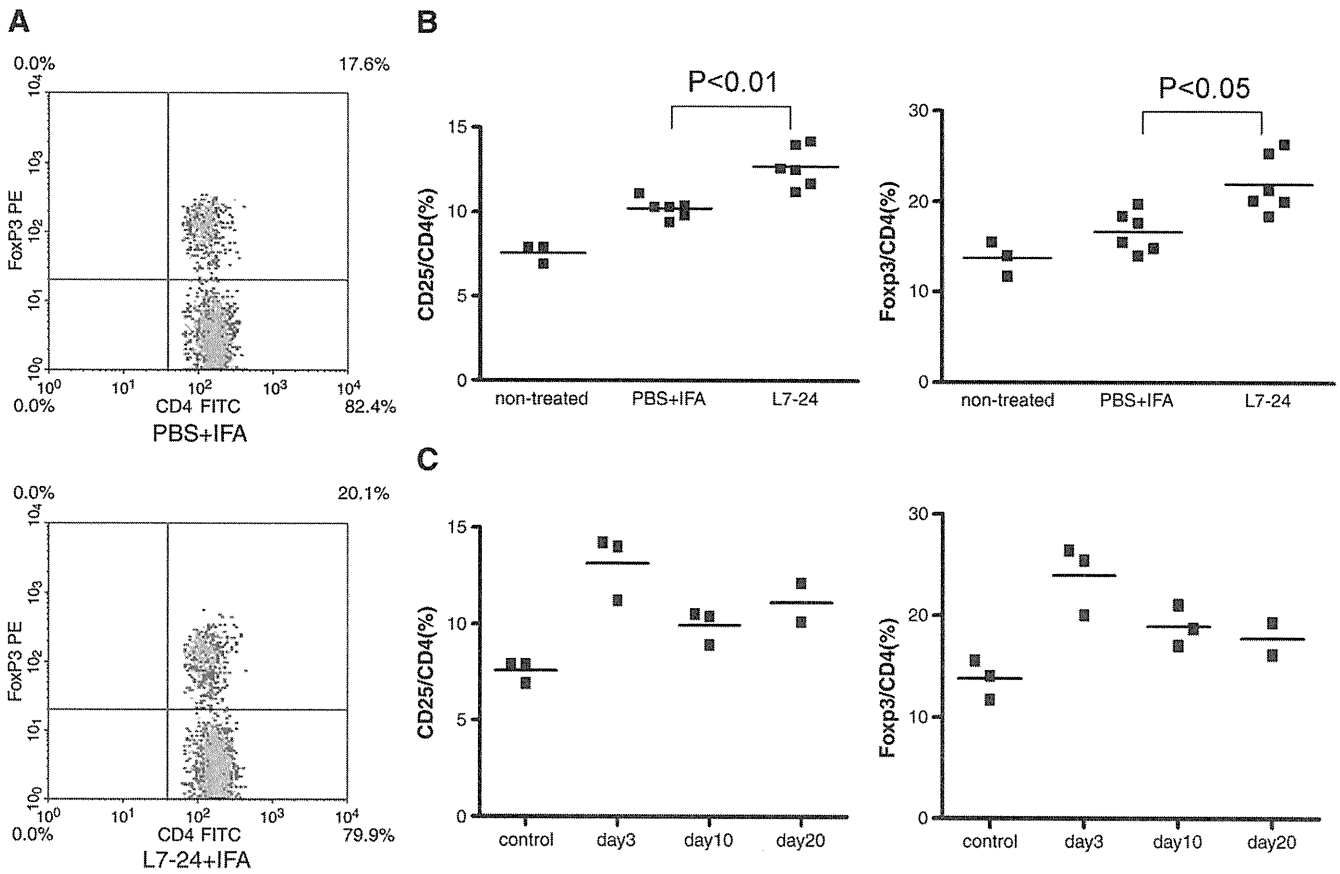

Figure 3 Regulatory T cells are induced by L7–24 peptide immunization. Ten- to 14-week-old NOD mice were immunized with 100 µg of L7–24 peptide twice weekly. The percentages of spleen and pancreatic lymph node cells were analysed by flow cytometry. The percentages of $CD25^+/CD4^+$ and $FoxP3^+/CD4^+$ T cells in spleen were analysed 3 days after the second administration (A and B). The change in the percentage of Tregs in the spleen from L7–24 peptide-immunized mice with time (C); the peak occurred on day 3.

The leader sequence is a peptide that is required for the entry of preproinsulin into the endoplasmic reticulum. This peptide is synthesized in an equimolar ratio to proinsulin, but it is not released outside β cells and is cleaved by a signal peptidase between L24 and B1 at the endoplasmic reticulum and processed inside β cells after separation from proinsulin. An 18-mer of the peptide from preproinsulin 7 to 24 (L7–24) was selected as a candidate for the therapeutic peptide.

Administration of L7–24 peptide stimulates a Th2 response in NOD mice

To investigate the therapeutic potential of this peptide, 100 μg of this peptide was emulsified in IFA and administered subcutaneously into the neck of 10- to 14-week-old NOD mice. Seven days after the last immunization, spleen cells were cultured with several peptides and IFN-γ and IL-4 secretion were measured using an ELISPOT assay. ConA induced strong response in both IFN-γ and IL-4 secretion. Immunization with L7–24 did not induce IFN-γ secretion but it induced IL-4 secretion significantly in response to L7–24 peptide (Figs. 1A and B). Interestingly, immunization with HEL10–23 peptide, which also binds to I-A^{g7}, induced the secretion of both IL-4 and IFN-γ in response to HEL10–23 peptide (Fig. 1C). The different results for HEL10–23 peptide and L7–24 peptide might be explained by the fact that HEL is not an autoantigen but a foreign antigen that the immune system of the NOD mice had not been exposed to before immunization.

IL-10 and TGF-β are also critical immunoregulatory cytokines that suppress autoimmune processes in various diseases. Supernatants from splenocytes of L7–24-immunized mice were cultured with antigenic peptides, and the concentrations of cytokines in the supernatants were measured by ELISA. L7–24 peptide immunization also induced upregulation of IL-10 secretion in response to L7–24 peptide (Fig. 2A). The amount of TGF-β in supernatant was not different from that of control mice (Fig. 2B).

Administration of L7–24 peptide can induce regulatory T cells

$CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) play a crucial role in suppressing autoimmune reactions. Tregs induced by a specific antigen have an antigen-specific immunoregulatory effect, which may be extended to include immunological responses to other antigens.

Three days after the second administration of L7–24 peptide, spleen cells were collected and $CD4^+CD25^+FoxP3^+$

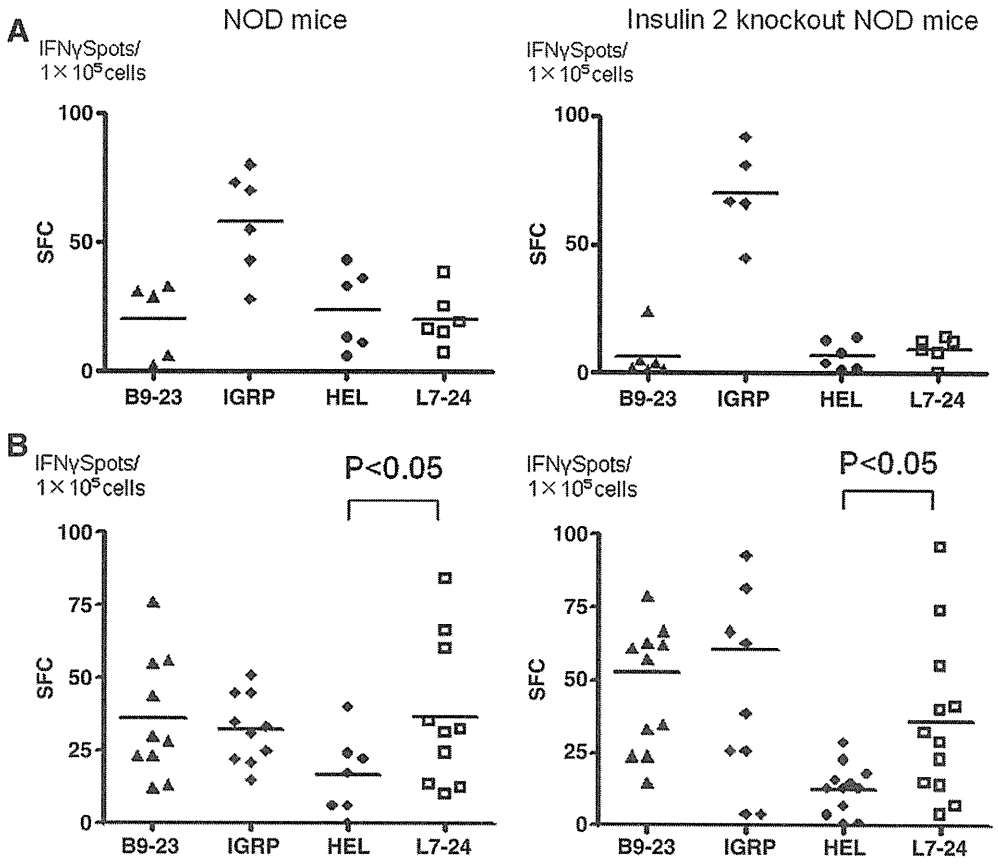

Figure 4 Spontaneous Th1 reaction to L7–24 peptide. Spleen cells and islet-infiltrating cells of 20- to 24-week-old NOD mice and 10–12-week-old insulin 2 knockout NOD mice were analysed by ELISPOT. (A) Ten thousand spleen cells were cultured for 48 h with each peptide. Secretion of IFN-γ in response to L7–24 was not observed. (B) IFN-γ spot forming colony (SFC) from islet-infiltrating cells was analysed. Islet-infiltrating cells of both NOD mice and insulin 2 knockout NOD mice showed vigorous production of IFN-γ in response to L7–24.

cells were analysed by flow cytometry. The percentages of $CD25^+/CD4^+$ and $FoxP3^+/CD4^+$ cells were significantly higher in L7–24 peptide-immunized mice than in controls (Figs. 3A and B). The percentage of cells peaked on the third day after the second injection (Fig. 3C). Unexpectedly, the percentage of Tregs in the pancreatic lymph node did not differ between L7–24 peptide-immunized mice and unimmunized mice (data not shown).

Islet-infiltrating cells of late insulitis secrete IFN-γ in response to L7–24 peptide

To examine whether the L7–24 sequence is targeted by pathogenic T cells, we analysed splenocytes and islet-infiltrating cells of untreated NOD mice (20 weeks old) and insulin 2 knockout NOD mice (12 weeks old). IFN-γ secretion was not upregulated in response to L7–24 in splenocytes (Fig. 4A). However, islet-infiltrating cells of both NOD mice and insulin 2 knockout NOD mice showed vigorous production of IFN-γ in response to L7–24 (Fig. 4B).

Administration of L7–24 peptide prevents the development of diabetes

To explore the effect of L7–24 peptide in preventing diabetes, we injected 100 μg of the peptide in IFA subcutaneously twice weekly into 12-week-old NOD mice. Only three of 22 NOD mice treated with the peptide developed diabetes at 45 weeks of age. The protection was significant compared with the mice given IFA without peptide ($p=0.046$, Fig. 5A). The insulin 2 knockout NOD mouse model is an accelerated model of autoimmune diabetes that starts developing diabetes around 10 weeks of age, and the cumulative incidence of diabetes reaches almost 100% [7]. Unlike NOD mice, injection of L7–24 into 8-week-old insulin 2 knockout NOD mice did not significantly delay the development of diabetes (Fig. 5B).

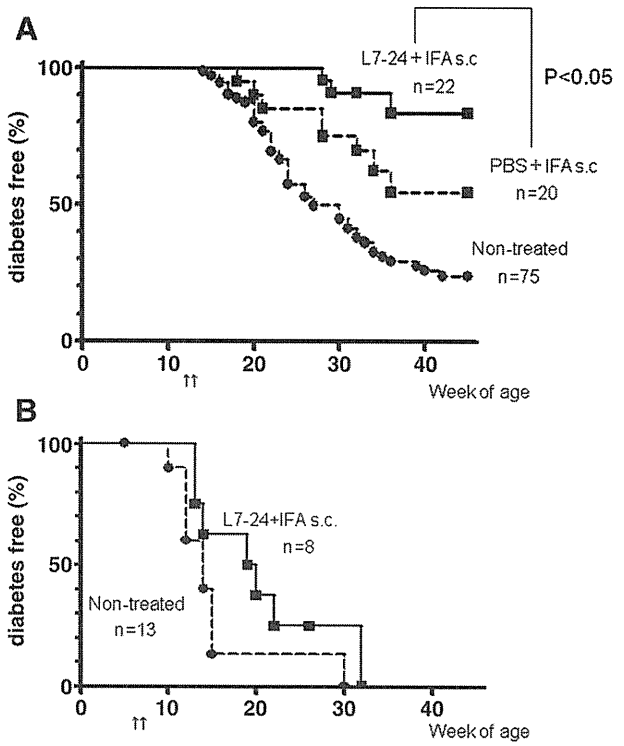

Figure 5 Administration of L7–24 peptide to 12-week-old NOD mice. (A) Twelve-week-old NOD mice were given two subcutaneous injections of L7–24 peptide. L7–24 peptide prevented diabetes compared with administration of IFA alone ($p=0.046$). (B) L7–24 peptide was administered similarly to insulin 2 knockout NOD mice. Only a slight delay in the onset of diabetes was observed. The little arrows indicate the timing of peptide immunization.

Administration of L7–24 peptide to diabetic NOD mice can restore normoglycemia

The therapeutic effect of L7–24 peptide on newly diabetic NOD mice was investigated next. Diabetic mice (BG >300 mg/dL) were assigned to receive injected L7–24, B9–23, or PBS only in IFA. After injections of the peptides twice a week, blood glucose concentration was monitored weekly. Five of 13 mice treated with L7–24 became normoglycemic 2–5 weeks after the last injection, whereas only one of 18 mice treated with PBS or B9–23 in IFA returned to normoglycemia (Figs. 6A–C). Histological examination of pancreata of the mice 16 weeks after restoration from hyperglycemia showed that some islets were preserved despite the remarkable infiltration of lymphocytes (Figs. 6D and E). Insulin and glucagon staining showed the retention of insulin-producing cells along with scattered glucagon-producing cells in the islets.

Discussion

The ultimate goal of treatment for autoimmune diabetes is antigen-specific suppression of the disease. There is increasing evidence that induction of antigen-specific Tregs is critical to halting the disease process [16,17]. Immunotherapy is currently applied in clinical practice to treat allergic diseases such as allergic rhinitis and asthma. However, the application of antigen-specific immunotherapy has not yet been successful in the treatment of autoimmune diseases.

There has been notable progress in immunotherapy for T1D. Subcutaneous injection of the GAD molecule with alum significantly reduces the progressive decline in β cell function without severe side effects [6,18,19]. Insulin is expected to be a promising autoantigen for the antigen-specific treatment of T1D. Several studies of NOD mice have shown the effectiveness of insulin administration in preventing the development of diabetes [20–22]. Although clinical studies of human T1D have been done, the clinical effectiveness of insulin administration has not been shown in humans, except for slight benefits in participants with a high titer of insulin autoantibody in the Diabetes Prevention Trial of T1D [10,11,23]. Nevertheless, insulin is undoubtedly the most abundant and specific molecule in the β cell, and there is increasing evidence that insulin is a primary antigen for T1D [24]. We hypothesized that additional epitopes besides those in the insulin A–B chain might be useful for providing tolerance in T1D, and we screened the sequence of the leader peptide that is removed from proinsulin in the rough

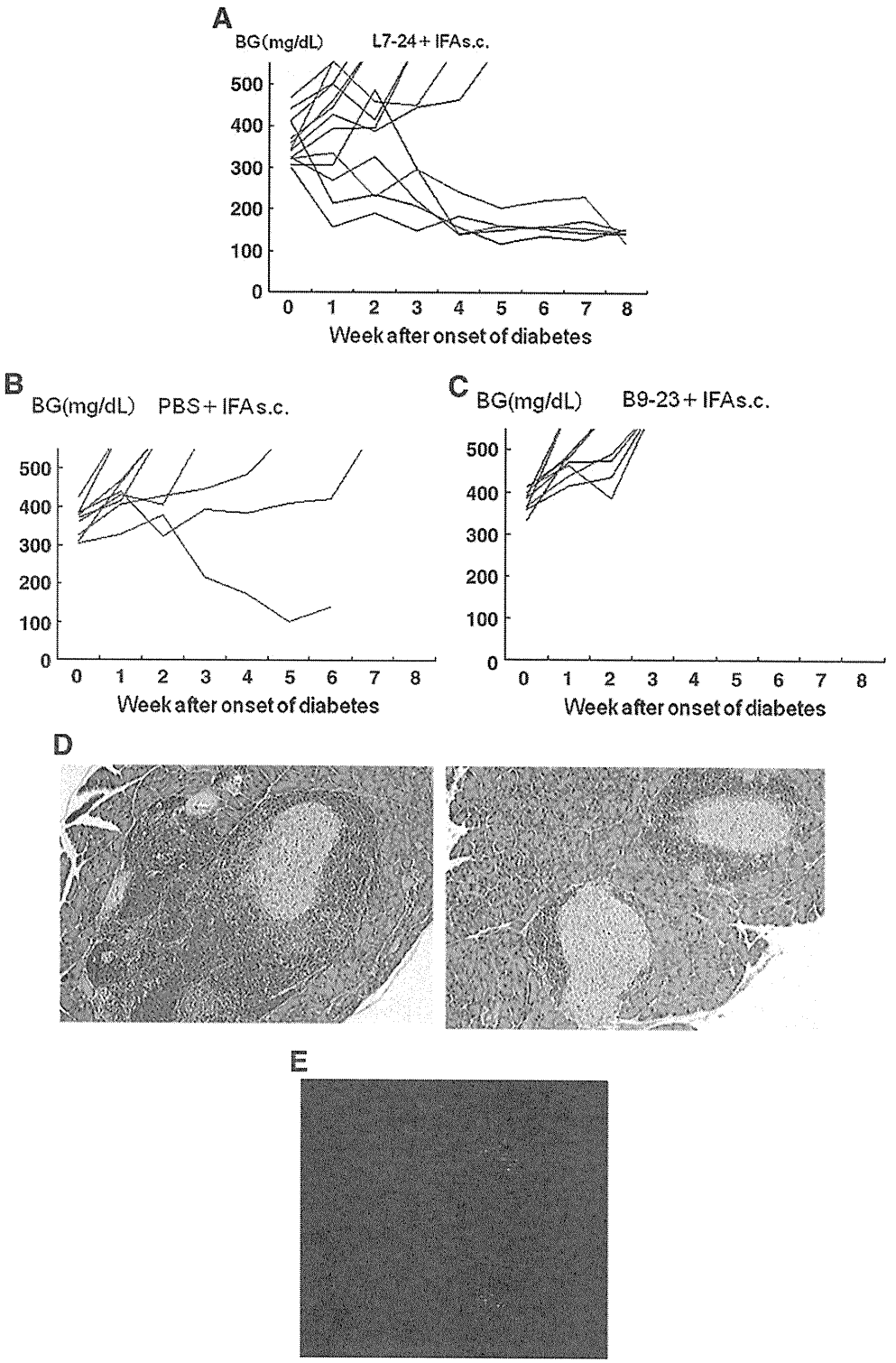

Figure 6 Administration of L7–24 to newly diabetic NOD mice. L7–24 peptide was administered to newly diabetic NOD mice (BG > 300 mg/dL). Five of 13 mice achieved remission of diabetes (A) whereas most mice did not by the administration of PBS in IFA (B) or B9–23 in IFA (C). Mean ± SD of blood glucose of each group on entry are as follows: L7–24, 362±53 mg/dl; PBS, 361±40 mg/dl; and B9–23, 380±26 mg/dl. Hematoxylin and eosin staining of islets of NOD mice that achieved remission show preservation of islets despite the marked lymphocyte infiltration (D). Insulin (red) and glucagon (green) staining showed the retention of insulin-producing cells along with scattered glucagon-producing cells in the islets (E).

endoplasmic reticulum before the entry into the secretory granule.

We found that L7–24 peptide increased the frequency of $CD25^{+}Foxp3^{+}$ Tregs and the production of regulatory cytokines such as IL-4 and IL-10. Administration of this peptide to 12-week-old NOD mice significantly reduced the incidence of diabetes, probably by stimulating regulatory cytokine-producing cells. The milder suppression of diabetic development in groups that received PBS with IFA was an unexpected observation. The protective effect of Freund's complete adjuvant (CFA) but not IFA to the development of diabetes in NOD mice is widely recognized [25]. There was, however, a report that showed the milder protective effect of IFA similar to our observation [26].

Interestingly, analysis of islet-infiltrating cells in late-stage insulitis demonstrated vigorous IFN-γ secretion in response to L7–24 peptide. The result suggests that L7–24 is supposed to be naturally processed and presented in the course of autoimmune diabetes in NOD mice. A study using pools of preproinsulin peptides showed an IL-2 response after subcutaneous administration of L7–24 peptide emulsified in Freund's complete adjuvant but not in IFA, although the Th2 responses were not reported [27]. In our study, L7–24 peptide immunization produced a Th2 cytokine profile due to immunization in the presence of IFA. This peptide may have potency for both the Th1 and Th2 responses, although Th2 immunogenicity can be induced more predominantly in IFA. Unlike L7–24, HEL10–24 in IFA seems to induce equally both Th1 and Th2 response. Therefore, L7–24 can be administered more safely than HEL, which induce adverse side effect by Th1 response.

It was advocated recently that self-peptides with low affinity for MHC are important as autoantigenic epitopes [28–30]. The hypothesized reason for this phenomenon is that autoreactive T cells that recognize a weak MHC-binding peptide can escape negative selection in the thymus. In contrast, T cells that react with stronger MHC-binding peptides are anticipated to receive negative selection in the thymus and to survive as natural Tregs in the periphery [31]. L7–24 was selected based on an alignment algorithm described by E.R. Unanue et.al [13]. The peptide has three amino acids that have a high affinity for pockets of the MHC $I\text{-}A^{g7}$ molecule and is expected to be present frequently in the thymus. Tregs induced with L7–24 peptide may be explained by the high binding affinity of this peptide to the $I\text{-}A^{g7}$ molecule.

Although L7–24 peptide showed a therapeutic effect, the effect remains insufficient to protect fully against the disease. Administration of the peptide to 12-week-old NOD mice significantly prevented diabetes, but some mice became diabetic even after immunization. In addition, L7–24 peptide could not prevent diabetes in insulin 2 knockout NOD mice, which develop diabetes at a faster rate than normal NOD mice. As disease progresses, more epitopes are being targeted due to epitope spreading. At advanced stages of the disease, combination of more than one epitopes may prove more beneficial. Combined peptide therapy might be an effective immunotherapy in the future, and the combination of peptide therapy and CD3 antibody may be also a promising option [32].

Insulin-derived epitopes are among the autoantigens specific to β cell and the most abundant epitopes in β cell. The reason why whole insulin immunization have not succeeded in protecting from type 1 diabetes (T1D) in humans is probably due to some issues that remain to be resolved, such as the use of correct dosage, more efficient immunization schemes and possibility of critical epitopes other than A-B chain.

The leader peptide of insulin has not attracted the attention of researchers as an autoantigen relevant to T1D. However, a recent study using human HLA-A0201 showed that the CD8 epitope of the leader peptide is a candidate autoantigen in human T1D [33]. Taken together, these previous findings and our results suggest that the leader sequence contains some critical determinants for development of diabetes in both human T1D and NOD mice. Our study is the first to demonstrate a therapeutic effect of the peptide, which is contained in the leader sequence. The identification of the T cell response to the leader sequence in patients with T1D suggests that some of the leader sequence may also be applicable in the protection against and cure for humanT1D.

Acknowledgements

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Regulatory CD8$^+$ T cells induced by exposure to all-trans retinoic acid and TGF-β suppress autoimmune diabetes

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ABSTRACT

Antigen-specific regulatory CD4$^+$ T cells have been described but there are few reports on regulatory CD8$^+$ T cells. We generated islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific regulatory CD8$^+$ T cells from 8.3-NOD transgenic mice. CD8$^+$ T cells from 8.3-NOD splenocytes were cultured with IGRP, splenic dendritic cells (SpDCs), TGF-β, and all-trans retinoic acid (ATRA) for 5 days. CD8$^+$ T cells cultured with either IGRP alone or IGRP and SpDCs in the absence of TGF-β and ATRA had low Foxp3$^+$ expression (1.7 ± 0.9% and 3.2 ± 4.5%, respectively). In contrast, CD8$^+$ T cells induced by exposure to IGRP, SpDCs, TGF-β, and ATRA showed the highest expression of Foxp3$^+$ in IGRP-reactive CD8$^+$ T cells (36.1 ± 10.6%), which was approximately 40-fold increase compared with that before induction culture. CD25 expression on CD8$^+$ T cells cultured with IGRP, SpDCs, TGF-β, and ATRA was only 7.42%, whereas CD103 expression was greater than 90%. These CD8$^+$ T cells suppressed the proliferation of diabetogenic CD8$^+$ T cells from 8.3-NOD splenocytes *in vitro* and completely prevented diabetes onset in NOD-scid mice in cotransfer experiments with diabetogenic splenocytes from NOD mice *in vivo*. Here we show that exposure to ATRA and TGF-β induces CD8$^+$Foxp3$^+$ T cells *ex vivo*, which suppress diabetogenic T cells *in vitro* and *in vivo*.

1. Introduction

The adaptive immune response is initiated by T cells that express receptors for diverse antigens. The repertoire of T cell receptors is regulated by positive selection and negative selection. Autoreactive T cells are usually deleted by negative selection in the thymus [1]. However this process is not exhaustive: T cells expressing receptors with low affinities for self-antigens are usually present in peripheral organs and may cause autoimmune disease if activated. Suppressor mechanisms for self-reactive T cells in peripheral organs have been presumed. The concept of T cell suppression of the immune response was first proposed by Gershon and Kondo for regulatory CD8$^+$ T cells [2]. There are few studies on regulatory CD8$^+$ T cells because of the difficulty of identifying regulatory CD8$^+$ T cell populations and their mechanisms of action. The CD4$^+$ population of regulatory T cells (Tregs) was first described by Sakaguchi et al. as CD4$^+$CD25$^+$ T cells [3]. Although CD25 expression has been used as a marker to identify Tregs, it is not specific for Tregs because it is expressed by all activated T cells. Recent studies revealed that the forkhead/winged helix family (Foxp3) transcription factor is a master switch specific to CD4$^+$CD25$^+$ Tregs that induces differentiation of naive T cells into the Treg lineage and maintains their suppressive function [4,5].

The CD4$^+$CD25$^+$ T cells described by Sakaguchi et al. originated in the thymus and developed their suppressive function in peripheral organs. On the other hand, Luo et al. revealed that transforming growth factor-β (TGF-β) and β-cell peptide-pulsed dendritic cells from NOD mice induced CD4$^+$CD25$^+$Foxp3$^+$ T cells from naïve T cells in BDC2.5-NOD splenocytes, which suppressed diabetes onset in cotransfer experiments [6]. In contrast to the regulatory cells in the thymus, BDC2.5 CD4$^+$CD25$^-$ T cells can be switched to CD4$^+$CD25$^+$Foxp3$^+$ T cells by regulatory cytokines such as TGF-β.

Vitamin A (retinol) and its metabolites (retinoids) are a group of potent natural or synthetic molecules which act as modulators for a variety of inflammatory and immunological events in immune system. The mechanism of this molecule has been reported as suppression of inflammatory immune cells, modulation of the function of immune cells and production of several cytokines [7]. All-trans retinoic acid (ATRA), a potent retinoids, has been clinically used to treat acute leukaemia and acne vulgaris [8,9]. Now it has also been reported as having a potential of generating CD4$^+$ Tregs [10,11]. In addition, dendritic cells purified from the small intestine were found to undergo a high level of CD4$^+$ Treg conversion when exposed to TGF-β and ATRA that was highly expressed in GALT [12].

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Recent publications have shown that the regulatory T cell family consists not only of $CD4^+$ T cells but also $CD8^+$ T cells [13–17], presuming that regulatory $CD8^+$ T cells can be induced from these naïve $CD8^+$ T cells in 8.3-NOD mice in an adequate milieu. In this study, we generated regulatory $CD8^+$ T cells from transgenic 8.3-NOD mice expressing CD8-TCR specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) in β-cells [18]. $CD8^+$ T cells exposed to ATRA and TGF-β exhibited regulatory functions *in vitro* and *in vivo*.

2. Materials and methods

2.1. Mice

NOD/Shi/Kbe mice were maintained at the Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan. The cumulative incidence of diabetes is 85% in females and 30% in males at 40 weeks of age. NOD-scid mice were purchased from Clea Japan (Osaka, Japan). 8.3-NOD mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were treated according to the Guidelines for Animal Experimentation of Kobe University School of Medicine.

2.2. Antibodies and reagents

FITC-conjugated anti-mouse monoclonal antibodies, CD8 (53–6.7) and CD103 (M290), were purchased from Pharmingen (San Diego, CA), as were the phycoerythrin (PE)-conjugated anti-mouse monoclonal antibodies, CD4 (L3T4), CD8 (53–6.7), and B220 (RA3–6B2), peridinin chlorophyll protein complex (PerCP)-conjugated anti-mouse CD4 monoclonal antibody (L3T4), and the APC-conjugated anti-mouse CD25 monoclonal antibody (3C7). The PE-conjugated anti-mouse Foxp3 monoclonal antibody (FJK-16s) was purchased from eBioscience (San Diego, CA). Anti-CD11c (N418) microbeads and the CD8 isolation kit were purchased from Miltenyi Biotec (Bergisch–Gladbach, Germany). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Dojindo (Kumamoto, Japan).

2.3. Cell purification and culture

Splenic dendritic cells (SpDCs) were purified from 8- to 12-week-old 8.3-NOD mice splenocytes using CD11c-magnetic beads. After selection using an autoMACS magnetic cell sorter (Miltenyi Biotec), $CD8^+$ T cells were purified by depletion of CD4-, B220-, CD49b-, CD11b-, and Ter-119-positive cells. To study the proliferation of $CD8^+$ T cells, some cells were stained with 10 μM CFSE for 15 min at 37 °C followed by two washes with HBSS at 4 °C. All cells were cultured in an atmosphere containing 5% CO_2 at 37 °C in 96-well tissue culture plates in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM sodium pyruvate, 10 mM Hepes buffer, 50 IU/ml penicillin, 50 μg/ml streptomycin, 40 μg/ml gentamycin and 5×10^{-5} M 2-mercaptoethanol. $CD8^+$ T cells (6.0×10^4) were cultured with 0.1 μM of IGRP alone or with IGRP and 2.0×10^4 of SpDCs as controls. In addition to IGRP and SpDCs, $CD8^+$ T cells in other groups were cultured with 2 ng/ml of TGF-β or with TGF-β and 10 nM of ATRA to induce the regulatory cells. Five-days later, cells were harvested and stained with several antibodies and propidium iodide (PI); only PI-negative cells were analyzed using the FACS 440 flow cytometer (Becton Dickinson, San Jose, CA). For each group, the $CD8^+$ T cells were cultured as described previously and harvested; only $CD8^+$ T cells were selected using the magnetic beads. $CD8^+$ T cells cultured with IGRP alone were designated I cells, those cultured with IGRP and SpDCs were designated ID cells, those cultured with IGRP, SpDCs, and TGF-β were designated IDT cells, and those cultured with IGRP, SpDCs, TGF-β, and ATRA were designated IDTA cells.

2.4. Proliferation assays

$CD8^+$ T cells from 8.3-NOD splenocytes that were selected using magnetic beads were stained with 10 μM CFSE for 15 min at 37 °C and were washed twice. Induced $CD8^+$ T cells (1.0×10^5) (I cells, ID cells, IDT cells, or IDTA cells) and CFSE-stained fresh $CD8^+$ T cells (1.0×10^5) were cultured together with 0.1 μM of IGRP. After 3 days, all cells were harvested and only $CFSE^+$ cells were analyzed using flow cytometry. To further examine the suppressor activity of induced $CD8^+$ T cells *in vitro*, freshly-isolated effector $CD8^+$ T cells (1.0×10^5) (E) were cultured with induced $CD8^+$ T cells (IDT cells or IDTA cells) (R) at the indicated ratios in the presence of 0.1 μM of IGRP.

2.5. Adoptive transfer

$CD8^+$ T cells (6.0×10^6) from 8.3-NOD splenocytes cultured with IGRP alone or IGRP, SpDCs, TGF-β, and ATRA were intravenously cotransferred into 8-week-old NOD-scid mice with 1×10^7 diabetogenic NOD splenocytes. The recipients were monitored for diabetes onset by testing urine glucose level twice weekly. Diabetes was defined as a blood glucose concentration greater than 250 mg/dl (13.9 mmol/l) on two consecutive days.

2.6. Statistical analysis

Statistical analysis of the incidence of diabetes was performed using the log-rank test. Statistical analyses of flow cytometric data were performed using the Mann–Whitney *U* test. A p value less than 0.05 was considered significant. All data are presented as the means ± SD.

3. Results

3.1. Induction of $CD8^+Foxp3^+$ T cells from splenocytes in 8.3-NOD mice

The prevalence of $CD8^+Foxp3^+$ T cells among splenocytes in 7-week-old 8.3-NOD mice was first analyzed using flow cytometry to evaluate "natural" $CD8^+Foxp3^+$ T cells. The population of $CD8^+$ T cells in the spleen from 8.3-NOD mice exceeded 50% of splenocytes, which was higher than that of littermate NOD mice (Fig. 1A). The population of $CD8^+Foxp3^+$ T cells in the spleen from 8.3-NOD mice was no more than 1.0% of splenocytes, which was equal to that in littermate NOD mice (Fig. 1B).

SpDCs and $CD8^+$ T cells were cultured for inducing regulatory $CD8^+$ T cells *ex vivo*. SpDCs were selected from the splenocytes of 8.3-NOD mice using CD11c-magnetic beads. $CD8^+$ T cells were negatively selected using magnetic beads followed by labeling with CFSE. CFSE-labeled $CD8^+$ T cells (6.0×10^4) were cultured for 5 days with 0.1 μM of IGRP alone (I cells), IGRP and 2.0×10^4 SpDCs (ID cells), IGRP, SpDCs, and 2 ng/ml of TGF-β (IDT cells) or with IGRP, SpDCs, TGF-β, and 10 nM of ATRA (IDTA cells). Fig. 1C and D show the *in vitro* proliferation of $Foxp3^+$ cells in cultured $CD8^+$ T cells from 8.3-NOD splenocytes. Only 1.7 ± 0.9% of I cells and 3.2 ± 4.5% of ID cells expressed Foxp3, whereas 8.6 ± 6.7% of IDT cells and 21.4 ± 4.2% of IDTA cells expressed Foxp3. IDTA cells showed the highest expression of Foxp3 and significantly higher expression than I cells ($^*p = 0.028$). To further examine whether the efficiency of conversion to Tregs can be increased, we also cultured $CD8^+$ T cells from 8.3-NOD mice with titrating concentrations of ATRA (0, 1, 5, 10, and 20 nM) in the presence of constant IGRP, splenic DC, and TGF-β concentrations. The percentage of $Foxp3^+$

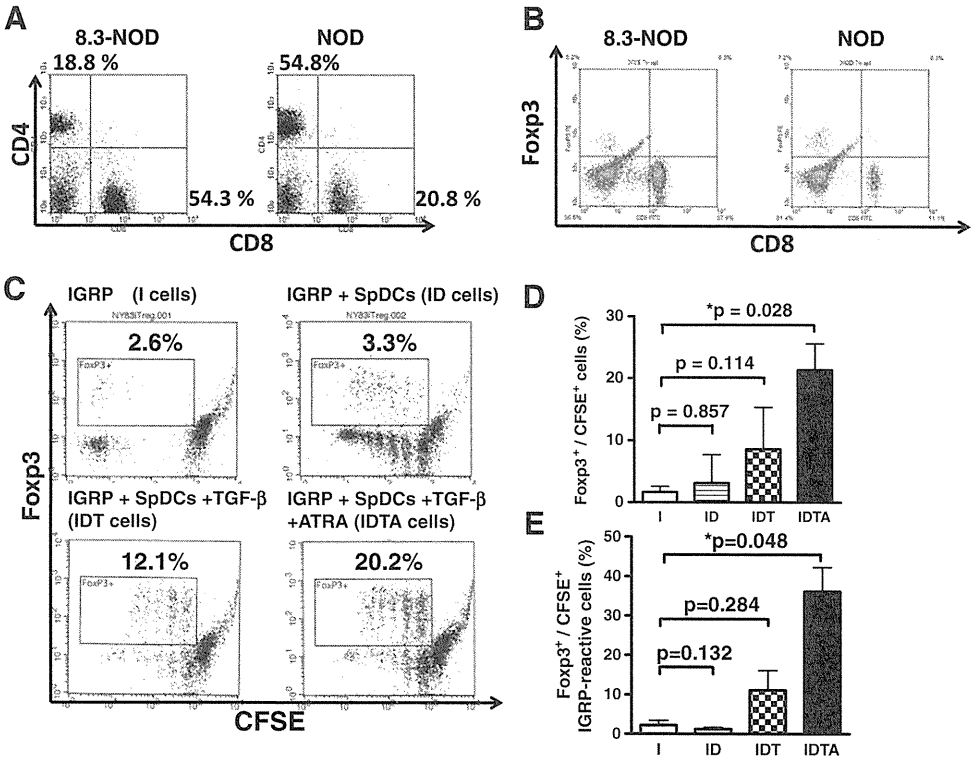

Fig. 1. (A, B) Splenocytes from 7-week-old 8.3-NOD or NOD mice were stained with anti-CD4 and CD8 antibodies (A) or anti-FoxP3 and CD8 antibodies (B). A representative example of four separate experiments is shown. (C, D, E) CFSE-labeled $CD8^+$ T cells (6.0×10^4) selected from the splenocytes of 7-week-old 8.3-NOD mice were cultured for 5 days with 0.1 μM of IGRP alone (I cells), IGRP and 2.0×10^4 SpDCs (ID cells), IGRP, SpDCs, and 2 ng/ml of TGF-β (IDT cells), or IGRP, SpDCs, TGF-β, and 10 nM of ATRA (IDTA cells). Five days later, all cells were harvested and gated on PI-negative and CFSE positive cells. A representative example (C), percentage of $Foxp3^+$ cells in $CFSE^+$ cells ($^*p = 0.028$, I vs. IDTA) (D) and percentage of $Foxp3^+$ cells in IGRP-reactive $CFSE^+$ cells ($^*p = 0.048$, I vs. IDTA) (E) are shown.

cells in 10 nM of ATRA was the highest among these titrating concentrations (data not shown). Among IGRP-responded $CD8^+$ T cells, 36.1 ± 10.6% of IDTA cells showed Foxp3 expression, which was approximately 40-fold increase compared with that before induction culture and significantly higher than that of I cells ($^*p = 0.048$) (Fig. 1E).

3.2. Cell surface marker of $CD8^+Foxp3^+$ T cells

To determine which surface markers were expressed on $CD8^+Foxp3^+$ T cells, IDT cells or IDTA cells were stained with CD25 and CD103 antibodies (Fig. 2). In contrast to $CD4^+Foxp3^+$ T cells, only some of the $CD8^+Foxp3^+$ T cells were CD25-positive, and most were CD103-positive.

3.3. In vitro suppression assay

To examine the suppressor activity of $CD8^+$ T cells *in vitro*, 1.0×10^5 of the CFSE-labeled effector $CD8^+$ T cells were cultured with IGRP and 1.0×10^5 of I, ID, IDT, or IDTA cells (Fig. 3A). Proliferation of $CD8^+$ T cells in the presence of I, ID, IDT, or IDTA cells with IGRP was compared with that induced by IGRP alone. % Suppression of each proliferation was −1.8 ± 7.6%, 2.9 ± 9.0%, 1.0 ± 8.5%, and 12.9 ± 8.9%, respectively, and the presence of I, ID, or IDT cells did not markedly suppress proliferation of IGRP-reactive $CD8^+$ T cells. In addition, there was no significant suppression in the presence of ID cells or IDT cells compared with the presence of I cells ($p > 0.05$). However, only the presence of IDTA cells significantly suppressed proliferation of IGRP-reactive effector $CD8^+$ T cells than the presence of I cells ($^*p = 0.009$) (Fig. 3B). Furthermore, the suppressive activity seems to be strengthened in a E:R ratio-dependent manner, though not all of IDTA cells express Foxp3 (Fig. 3C).

3.4. In vivo suppression assay

Because IDTA cells alone have suppressor activity for diabetogenic antigen-specific $CD8^+$ T cells *in vitro*, *in vivo* suppressor activity was examined using a diabetes-transfer model. IDTA cells or I cells were intravenously transferred into NOD-scid mice with diabetogenic splenocytes from NOD mice. All of five mice injected with diabetogenic NOD splenocytes alone and six mice injected with diabetogenic splenocytes and I cells became diabetic by 44 days after transfer. In contrast, none of the five mice injected with diabetogenic splenocytes and IDTA cells became diabetic in this cotransfer experiment ($^*p = 0.01$, IDTA vs. control) (Fig. 4). In another set of experiment, none of mice injected with diabetogenic splenocytes and IDTA cells became diabetic even at 150 days after transfer which was more than 30 days after last positive control became diabetic (data not shown). These findings suggest that $CD8^+$ T cells induced by exposure to SpDCs, TGF-β, and ATRA have suppressor activity against the autoimmune response *in vivo* and that the disease should be suppressed only by the $Foxp3^+$ population.

4. Discussion

This study demonstrates that regulatory $CD8^+$ T cells can be induced from diabetogenic 8.3 transgenic NOD mice, which express the TCR-α and TCR-β of a diabetogenic H-$2K_d$-restricted β-cell cytotoxic $CD8^+$ T cell clone and promote diabetes [19]. Original NY8.3 $CD8^+$ cloned T cells cause severe insulitis and diabetes when cotransferred with NOD $CD4^+$ T cells [20]. The 8.3-NOD mice in our colony develop diabetes more rapidly than the original NOD mice, but the incidence of diabetes does not differ from that of NOD mice (75% among females aged 20 weeks and 30 weeks, respectively). Santamaria et al. reported that high avidity of 8.3 TCR T cells devel-

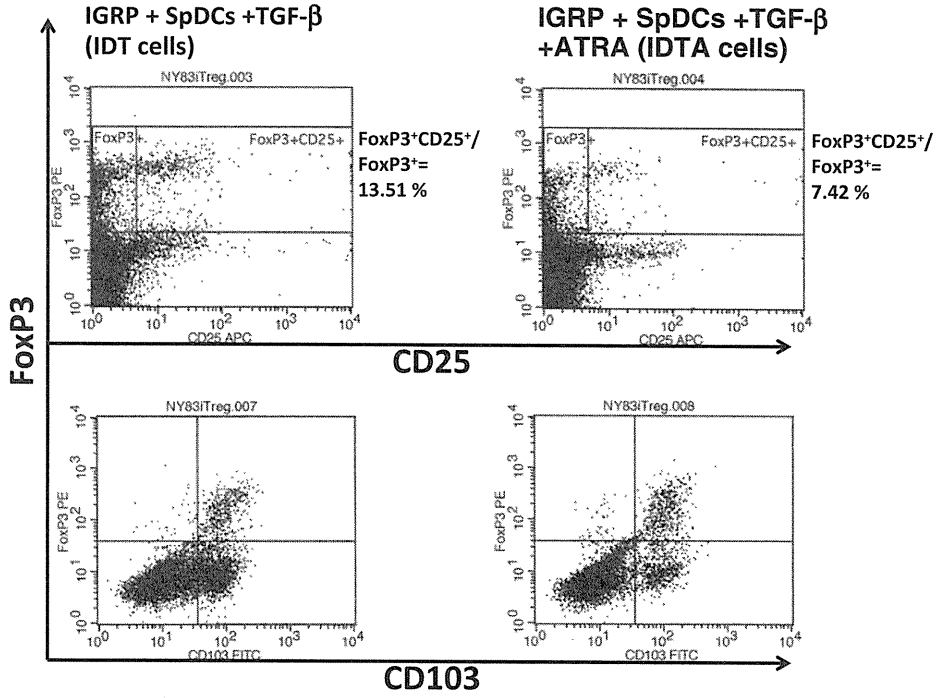

Fig. 2. IDT cells and IDTA cells were stained with antibodies against Foxp3, CD25, and CD103. Only some of the $CD8^+Foxp3^+$ T cells were CD25-positive, and most were CD103-positive. A representative example of four separate experiments is shown.

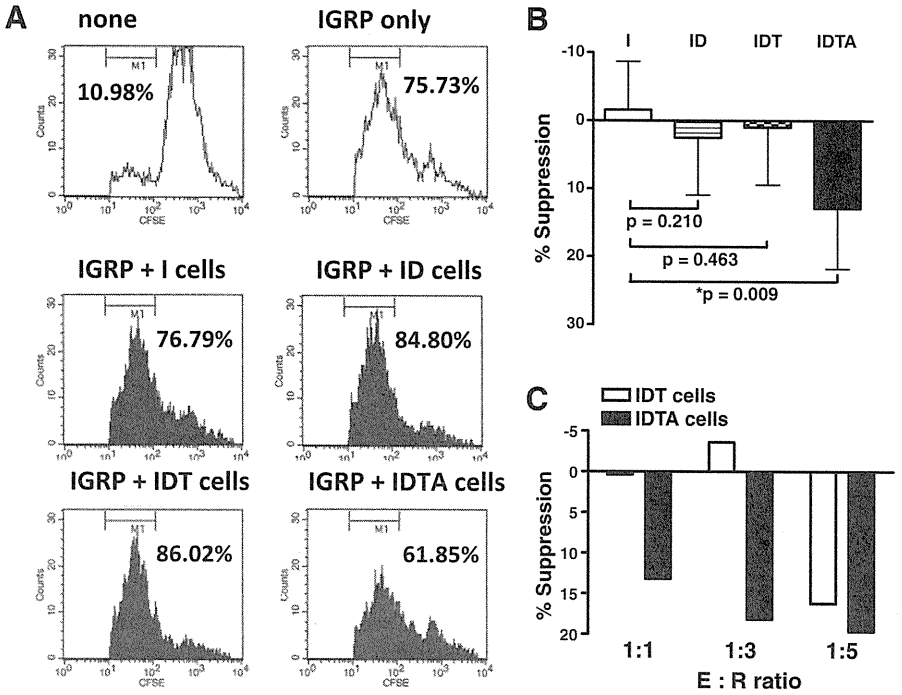

Fig. 3. CFSE-labeled $CD8^+$ T cells (1.0×10^5) purified from 8.3-NOD splenocytes were cultured for 5 days with IGRP alone or with IGRP and 1.0×10^5 induced $CD8^+$ T cells in each group shown in Fig. 1. Three days later, all cells were harvested and gated on $CFSE^+$ cells. (A) Proliferation of $CD8^+$ T cells induced by IGRP alone or in the presence of I, ID, IDT, or IDTA cells with IGRP was examined. A representative example of eight separate experiments is shown. (B) Proliferation of effector $CD8^+$ T cells in the presence of I, ID, IDT, or IDTA cells with IGRP was shown as % suppression, in comparison with that induced by IGRP alone ($^*p = 0.009$, I vs. IDTA). (C) Proliferation of effector $CD8^+$ T cells (E) in the presence of IDT, or IDTA cells (R) with IGRP in comparison with that induced by IGRP alone was shown as % suppression at the indicated E:R ratios.

ops with aging in 8.3-NOD mice [21]. Because 10–20% of the mice did not develop diabetes, we presumed that regulatory T cells were present in 8.3-NOD mice. We generated Tregs in $CD8^+$ T cells for this reason.

Induced regulatory $CD8^+$ T cells have been used in some studies [14–17], two of which showed that Foxp3 is an important marker of regulatory $CD8^+$ T cells [14,17]. Regulatory $CD8^+$ T cells cultured with IGRP, SpDCs, TGF-β, and ATRA showed suppressor activity and the highest expression of Foxp3, which indicates that $CD8^+Foxp3^+$ T cells played a key role in the generation of regulatory $CD8^+$ T cells. Although the role of the expression of Foxp3 in these $CD8^+$ T cells is not well understood, Foxp3 expression is known to induce regulatory T cells. The Foxp3-transduced 6426 $CD8^+$ T cell clone, which recognizes insulin B chain peptide 15–23, delayed the

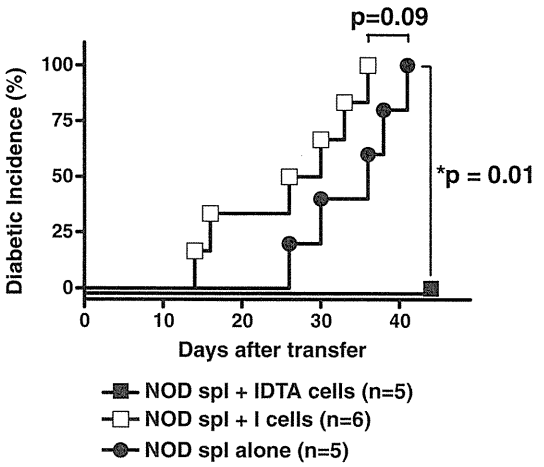

Fig. 4. Diabetogenic NOD splenocytes (1×10^7) alone (closed circle, $n = 5$) or with 6.0×10^6 I cells (open square, $n = 6$) or IDTA cells (closed square, $n = 5$) were transferred into 7- to 8-week-old NOD-scid mice. I cells had no effect on suppression of diabetes transfer, whereas IDTA cells completely suppressed diabetes transfer ($^{*}p = 0.01$, IDTA vs. control).

onset of diabetes compared with control 6426 clone when transferred into NOD-scid or young NOD mice [22]. This study indicated that expression of Foxp3 changes effector $CD8^+$ T cells into regulatory $CD8^+$ T cells.

Rigorous purification of regulatory $CD8^+Foxp3^+$ T cells in cellular transfusion material would prevent autoimmune diabetes. Because Foxp3 is not a surface marker, we examined whether the $CD8^+Foxp3^+$ T cells express specific surface markers that would enable purification of these cells *in vitro*. Unlike the case with $CD4^+CD25^+Foxp3^+$ T cells, CD25 is not a marker of regulatory $CD8^+$ T cells. More than 90% of the $CD8^+Foxp3^+$ T cells induced in our study also expressed CD103. CD103, the αEβ7 integrin, is a marker for alloantigen-induced regulatory $CD8^+$ T cells [15,23,24]. As $CD8^+Foxp3^-$ T cells also expressed CD103, it is not completely specific for $CD8^+Foxp3^+$ T cells. Purification of regulatory T cells using a CD103 antibody may isolate $CD8^+Foxp3^+$ T cells more efficiently.

ATRA has been reported to play an important role in immune system so far. It has been currently used to treat acute leukaemia and acne vulgaris [8,9]. The role of ATRA in the generation of $CD4^+$ regulatory T cells has also been reported recently [10–12]. Belkaid et al. reported that naïve $CD4^+Foxp3^-$ T cells converted $CD4^+Foxp3^+$ T cells in the gut and that gut-resident DCs produced ATRA. This conversion of Tregs occurred in a TGF-β and ATRA-dependent fashion [12]. Powrie et al. also reported that ATRA-dependent naïve T cells converted to Tregs after oral administration of antigen [11]. $CD4^+Foxp3^-$ T cells cultured with TGF-β, IL-2, and ATRA can convert into a $CD4^+Foxp3^+\alpha E\beta7^+CCR9^+$ phenotype *in vitro* and ATRA enhances the expression of Foxp3 and increases their suppressor activity [10]. ATRA can induce regulatory $CD4^+$ T cells from naïve $CD4^+$ T cells more efficiently in a Foxp3-dependent way. In this study, we first showed that ATRA and TGF-β was used to generate regulatory $CD8^+$ T cells *ex vivo*.

5. Conclusions

ATRA and TGF-β induce antigen-specific regulatory $CD8^+$ T cells in autoimmune diabetic mice. Regulatory $CD8^+$ T cells induced *ex vivo* would be useful as a therapeutic tool for autoimmune diabetes.

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