Table 3 | Relationships between Pro198Leu polymorphism of glutathione peroxidase 1 gene and quantitative neurological functions evaluated by multiple regression analysis

Independent variables	Model 1 dependent variables							Model 2 dependent variables						
	Vibration	Autonomic functions		Nerve conduction parameters				Vibration	Autonomic functions		Nerve conduction parameters			
	QVP	CVR–R	ΔBP	MCV	CMAP	SCV	SNAP	QVP	CVR–R	ΔBP	MCV	CMAP	SCV	SNAP
R^2 (*P*-value)	R^2 = 0.182 (*P* < 0.0001)	R^2 = 0.202 (*P* < 0.0001)	R^2 = 0.143 (*P* = 0.0012)	R^2 = 0.101 (*P* = 0.0254)	R^2 = 0.049 (*P* = 0.3987)	R^2 = 0.093 (*P* = 0.0522)	R^2 = 0.286 (*P* < 0.0001)	R^2 = 0.320 (*P* < 0.0001)	R^2 = 0.288 (*P* < 0.0001)	R^2 = 0.266 (*P* < 0.0001)	R^2 = 0.254 (*P* < 0.0001)	R^2 = 0.083 (*P* = 0.1588)	R^2 = 0.182 (*P* = 0.0005)	R^2 = 0.419 (*P* < 0.0001)
	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)
Age (years)	**0.281** (**0.0004**)	**−0.296** (**0.0003**)	0.079 (0.3281)	−0.121 (0.1445)			**−0.326** (**<0.0001**)	**0.288** (**<0.0001**)	**−0.303** (**<0.0001**)	0.088 (0.2425)	−0.133 (0.0823)		−0.080 (0.3241)	**−0.338** (**<0.0001**)
Sex (female: 0, male: 1)	0.062 (0.3950)	0.049 (0.5027)	0.063 (0.4036)	−0.142 (0.0681)			**−0.169** (**0.0157**)	0.072 (0.2984)	0.064 (0.3781)	0.048 (0.5058)	−0.093 (0.2049)		−0.025 (0.7484)	**−0.164** (**0.0122**)
Duration (years) (≥5: 0. 6–15: 1, 16≤: 2)	0.114 (0.1398)	**−0.215** (**0.0015**)	0.178 (0.0247)	−0.061 (0.4510)			**−0.265** (**0.0004**)	−0.062 (0.4192)	−0.118 (0.1406)	0.014 (0.8588)	0.100 (0.2184)		0.084 (0.3350)	−0.089 (0.2228)
Hypertension (no: 0, yes: 1)	0.120 (0.1149)	0.040 (0.5958)	**0.200** (**0.0109**)	−0.064 (0.4235)			−0.069 (0.3355)	0.058 (0.4249)	0.105 (0.1619)	0.122 (0.1052)	0.045 (0.5568)		−0.099 (0.2235)	0.004 (0.9516)
Dyslipidemia (no: 0, yes: 1)	−0.071 (0.3417)	0.037 (0.6153)	−0.129 (0.0899)	0.032 (0.6847)			0.115 (0.1032)	−0.016 (0.8112)	0.006 (0.9343)	−0.090 (0.2120)	0.001 (0.9954)		−0.011 (0.8882)	0.067 (0.3026)
Glycemic control (~fair: 0, poor: 1)	−0.007 (0.9271)	−0.103 (0.1572)	0.104 (0.1601)	**−0.191** (**0.0135**)			−0.089 (0.1923)	0.025 (0.7038)	−0.117 (0.0938)	0.123 (0.0791)	**−0.196** (**0.0062**)		**−0.246** (**0.0014**)	−0.101 (0.1093)
BMI (kg/m^2) (>22: 0. 22–25: 1, 25<: 2)	−0.072 (0.3443)	0.071 (0.3489)	**−0.205** (**0.0091**)	**0.164** (**0.0429**)			−0.091 (0.2088)	−0.063 (0.3725)	0.062 (0.3891)	**−0.197** (**0.0074**)	**0.163** (0.0286)		0.145 (0.0671)	−0.092 (0.1605)
GPx-1 genotype (Pro/Pro: 0, Pro/Leu: 1)	**0.176** (**0.0145**)	0.036 (0.6111)	−0.111 (0.1292)	0.047 (0.5339)			−0.044 (0.5124)	**0.154** (**0.0196**)	0.050 (0.4617)	−0.129 (0.0596)	0.060 (0.3849)		−0.009 (0.8987)	−0.021 (0.7360)
Retinopathy (no: 0, simple: 1, PPDR~: 2)								**0.455** (**<0.0001**)	**−0.306** (**0.0006**)	**0.380** (**<0.0001**)	**−0.320** (**0.0003**)		**−0.298** (**0.0017**)	**−0.409** (**<0.0001**)
Proteinuria (no:0, intermittent: 1, persistent: 2)								−0.091 (0.2517)	−0.041 (0.6236)	0.028 (0.7315)	**−0.181** (**0.0322**)		−0.068 (0.4482)	−0.001 (0.9862)

The significant regression formula on compound muscle action potential (CMAP) and sensory nerve velocity (SCV) were not obtained in model 1. The significant regression formula on CMAP was not obtained in model 2. β, Standard regression coefficient; BMI, body mass index; ΔBP, orthostasis-induced decreases in systolic blood pressure at standing; QVP, quantitative vibratory perception thresholds; CVR–R, coefficient of variation of RR intervals on electrocardiogram after 15 min resting; GPx-1, glutathione peroxidase 1 gene; MCV, motor nerve conduction velocity; PPDR, preproliferative diabetic retinopathy; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential, R^2, decision coefficient. Statistically significant *P*-value was shown by boldfaced type.

conduction parameters was proven (model 1). Virtually the same result was obtained from the reanalysis to which proteinuria and retinopathy were added as independent variables (model 2).

Table 4 shows the result of the multiple logistic regression and multiple regression analyses that contains a history of MVD as an independent variable (model 3). As in the results of model 1 and 2, significant associations of the GPx-1 genotype with DSPN, painful leg cramp and QVP were also observed in this model.

DISCUSSION

In the present study, we showed the following four major findings. First, the frequency of Pro/Leu type of the GPx-1 genotype in diabetic patients with DSPN was significantly higher than that in the patients without DSPN. Second, the frequencies of painful leg cramp, DSPN and impaired QVP in the patients with Pro/Leu type were significantly higher than those in the patients with Pro/Pro type, respectively. Third, Pro/Leu type was a significant risk factor associated with painful leg cramp, DSPN and history of MVD, but it was not associated with DAN. Fourth, though Pro/Leu type was a significant exacerbation factor of QVP, it had no association with other neurological functions.

Our first finding is that there is a significantly higher frequency of the Pro/Leu type in diabetic patients with DSPN compared with those without DSPN. The genotype frequency (%) of the Pro/Leu type in the Japanese population is quite similar to the present study (15.1 vs 13.9)[19]. So, Pro198Leu polymorphism of the GPx-1 gene seems to be relevant to the development of diabetic complications, but not to the onset of diabetes itself.

Our second finding indicates the possible relationship between the GPx-1 genotype and several manifestations of diabetic neuropathy by univariation analysis. The diabetic patients with Pro/Leu type were susceptible to impaired QVP in the toe, DSPN and painful leg cramps.

Our third and fourth findings confirmed this by multivariate analysis. The statistically significant associations between the GPx-1 genotype and DSPN, painful leg cramp, history of MVD and QVP impairment did not disappear, even if it was adjusted for microangiopathies (proteinuria and retinopathy), which are closely related to diabetic neuropathy[20]. The associations between the GPx-1 genotype and DSPN, painful leg cramp and QVP impairment also kept statistical significance, even after the adjustment for the history of MVD.

On the other hand, we could not observe any significant relationship between the GPx-1 genotype and DAN, autonomic and nerve conduction functions. In general, DSPN and DAN are considered to reflect mainly the large and small diameter nerve fiber dysfunctions, respectively[21]. Thus, the lack of association of the GPx-1 genotype with DAN and autonomic functions might show that the etiological factors of DSPN differ from those of DAN.

In contrast, impairment of nerve conduction is thought to be a reliable marker of DSPN. The amplitude and conduction abnormalities are most prominent in the distal segments of nerves in the legs; the potential for sensory nerve action in the sural nerve is especially sensitive and useful in identifying early abnormalities[22]. As we did not carry out nerve conduction studies in the lower limbs, accurate nerve conduction functions seemed not to be evaluated sufficiently in our study. Associations between the GPx-1 genotype and nerve conduction data in the lower limbs might provide different results. A more plausible explanation of this issue is that the GPx-1 genotype is mainly associated with QVT impairment, which is a part of the manifestation of DSPN. Actually, our data showed a strong association between the GPx-1 genotype and QVP, whereas a significant association with the GPx-1 genotype, diminished ATR and sensory symptoms was not proven. QVP reflects the functions of the peripheral and central nervous system, and it can be impaired by causes other than neuropathy, such as peripheral arterial disease. However, we suppose that the GPx-1 genotype affects the peripheral nerve function of vibratory sensation to some degree, because the patients with clinical peripheral arterial disease were excluded from the present study and the association of the GPx-1 genotype with QVP was independent from the history of MVD. Furthermore, a recent study using DCCT/EDIC participants proved that QVP is a sensitive measure of peripheral neuropathy[23]. Considering all of the aforementioned findings, we might be able to conclude that Pro198Leu polymorphism of the GPx-1 gene might be a candidate for the common genetic predisposition to MVD and DSPN, especially with an impairment of vibratory perception.

Two possible pathophysiological mechanisms of the association between the GPx-1 genotype and QVP impairment can be considered. One possible mechanism is impaired microcirculation in the peripheral nerve caused by vascular endothelial dysfunction elicited through accelerated oxidative stress in patients with a Pro/Leu genotype. We have previously reported that anti-oxidative activity of GPx-1 decreased in the Pro/Leu genotype[9]. Significant relationships between the GPx-1 genotype and painful leg cramps might support this possibility, because painful leg cramps are considered to reflect a circulatory disturbance in the leg and are frequently experienced in cold ischemic conditions. Another possible mechanism is direct nerve damage as a result of elevated oxidative stress. Neurotoxicity of excessive oxidative stress is widely recognized in experimental diabetic neuropathy. At present, the precise mechanism of the harmful effects of Pro/Leu genotype of the GPx-1 gene on QVT impairment is uncertain.

We have also shown a significant relationship between the GPx-1 genotype and the prevalence of painful leg cramps. Because painful leg cramps can be associated with various disorders, such as neurological, muscular, metabolic, endocrine and vascular diseases, a significant association between the GPx-1 genotype and painful leg cramps might not reflect DSPN. Furthermore, the prevalence of painful leg cramps occurring in self-administered questionnaires in 1524 diabetic patients under a primary care physician (25.5%) was not different from that in 501 non-diabetic subjects (29.4%) who underwent a corporate health screening examination (Nakatani M, Sasaki H, Kurisu S, Yamaoka H, Matsuno S, Ogawa K, Yamasaki H, Wakasaki H, Furuta H, Nishi M, Akamizu T, Nanjo K, 2011, unpublished

Table 4 | Relationships between glutathione peroxidase 1 gene polymorphism and subtype of diabetic neuropathy, painful leg cramp and quantitative neurological functions evaluated by multiple logistic regression and multiple regression analysis

Independent variables	Model 3 dependent variables			Model 3 dependent variables						
	Subtypes of diabetic neuropathy		Painful leg cramp	Vibration	Autonomic functions		Nerve conduction parameters			
	DSPN	DAN		QVP	CVR–R	ΔBP	MCV	CMAP	SCV	SNAP
R^2 (*P*-value)	R^2 = 0.210 (*P* < 0.0001)	R^2 = 0.530 (*P* < 0.0001)	R^2 = 0.103 (*P* = 0.0289)	R^2 = 0.320 (*P* < 0.0001)	R^2 = 0.303 (*P* < 0.0001)	R^2 = 0.266 (*P* < 0.0001)	R^2 = 0.254 (*P* < 0.0001)	R^2 = 0.093 (*P* = 0.1411)	R^2 = 0.189 (*P* = 0.0006)	R^2 = 0.419 (*P* < 0.0001)
	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	Adjusted OR (95% CI) *P*-value	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)	β (*P*-value)
Age (years)	1.010 (0.974–1.048) 0.5938	1.002 (0.933–1.076) 0.9619	0.995 (0.959–1.032) 0.7785	**0.292 (0.0001)**	**−0.278 (0.0003)**	0.091 (0.2337)	−0.135 (0.0833)	–	−0.096 (0.2446)	**−0.342 (<0.0001)**
Sex (female: 0, male: 1)	1.095 (0.514–2.335) 0.8136	1.904 (0.455–7.969) 0.3781	0.468 (0.217–1.010) 0.0529	0.076 (0.2664)	0.090 (0.2177)	0.051 (0.4859)	−0.095 (0.2033)	–	−0.039 (0.6258)	**−0.168 (0.0122)**
Duration (years) (≧5: 0. 6–15: 1, 16≦: 2)	1.343 (0.794–2.269) 0.2713	0.976 (0.408–2.336) 0.9567	1.139 (0.652–1.989) 0.6473	−0.062 (0.4217)	−0.118 (0.1386)	0.014 (0.8577)	0.100 (0.2203)	–	0.083 (0.3434)	−0.089 (0.2237)
Hypertension (no: 0, yes: 1)	2.115 (0.956–4.677) 0.0644	2.598 (0.708–9.531) 0.1499	0.742 (0.333–1.654) 0.4650	0.058 (0.4217)	0.108 (0.1472)	0.123 (0.1046)	0.044 (0.5625)	–	−0.105 (0.1978)	0.003 (0.9595)
Dyslipidemia (no: 0, yes: 1)	**0.466 (0.218–0.996) 0.0486**	0.833 (0.206–3.370) 0.7979	0.516 (0.241–1.102) 0.0875	−0.017 (0.8013)	−0.003 (0.9711)	−0.091 (0.2092)	0.001 (0.9866)	–	−0.007 (0.9303)	0.068 (0.2982)
Glycemic control (∼fair: 0, poor: 1)	1.731 (0.821–3.650) 0.1494	**9.470 (1.968–45.577) 0.0050**	1.412 (0.672–2.985) 0.3600	0.025 (0.7139)	−0.125 (0.0725)	0.122 (0.0823)	**−0.195 (0.0066)**	–	**−0.239 (0.0019)**	−0.100 (0.1148)
BMI (kg/m^2) (>22: 0. 22–25: 1, 25<: 2)	0.862 (0.545–1.364) 0.5263	**0.159 (0.055–0.457) 0.0007**	0.978 (0.621–1.540) 0.9235	−0.061 (0.3870)	0.072 (0.3212)	**−0.196 (0.0082)**	**0.162 (0.0308)**	–	0.135 (0.0894)	−0.093 (0.1569)
GPx-1 genotype (Pro/Pro: 0, Pro/Leu: 1)	**3.286 (1.156–9.346) 0.0257**	0.352 (0.042–2.965) 0.3366	**4.469 (1.725–11.578) 0.0021**	**0.157 (0.0194)**	0.064 (0.3454)	−0.126 (0.0696)	0.058 (0.4070)	–	−0.025 (0.7420)	−0.023 (0.7082)
Proteinuria (no: 0, intermittent: 1, persistent: 2)	0.692 (0.379–1.207) 0.1948	0.670 (0.299–1.503) 0.3314	1.529 (0.869–2.691) 0.1408	−0.090 (0.2624)	−0.030 (0.7158)	0.030 (0.7192)	**−0.182 (0.0322)**	–	−0.081 (0.3672)	−0.003 (0.9682)
Retinopathy (no: 0, simple: 1, PPDR∼: 2)	**2.921 (1.764–4.835) <0.0001**	**38.537 (5.553–267.423) 0.0002**	0.655 (0.384–1.115) 0.1187	**0.455 (<0.0001)**	**−0.307 (0.0006)**	**0.380 (<0.0001)**	**−0.320 (0.0004)**	–	**−0.293 (0.0020)**	**−0.409 (<0.0001)**
History of MVD (no: 0, yes: 1)	1.042 (0.279–3.653) 0.9491	0.890 (0.82–9.632) 0.9235	1.438 (0.421–4.909) 0.5621	−0.018 (0.7950)	−0.128 (0.0736)	−0.018 (0.3834)	0.012 (0.8722)	–	0.086 (0.2765)	0.0181 (0.7868)

The significant regression formula on compound muscle action potential (CMAP) were not obtained in model 3. β, Standard regression coefficient; BMI, body mass index; ΔBP, orthostasis-induced decreases in systolic blood pressure at standing; CI, confidence interval; CVR-R, coefficient of variation of RR intervals on electrocardiogram after 15 min resting; DAN, diabetic autonomic neuropathy; DSPN, distal symmetric polyneuropathy; MCV, motor nerve conduction velocity; MVD, macrovascular disease, OR, odds ratio; PPDR, preproliferative diabetic retinopathy; QVP, quantitative vibratory perception thresholds; R^2, decision coefficient; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential. Statistically significant *P*-value was shown by boldfaced type.

data). Therefore, the observed relationship between painful leg cramps and the Pro/Leu genotype might not be exclusively confined to a diabetic population.

As for common risk factors of MVD and diabetic neuropathy, several investigators reported common risk factors, such as obesity, dyslipidemia and hypertension. Most of the reports were epidemiological studies, and diabetic neuropathy was correspondent to the DSPN of the present study, though autonomic or nerve conduction functions were not carefully evaluated[3–5]. It might be speculated that the GPx-1 gene polymorphism could affect the increasing prevalence of DSPN though the deteriorating effect of vibratory perception.

Further studies, such as a prospective observational study, are necessary to confirm the association of the Pro198Leu polymorphism of the GPx-1 gene with diabetic neuropathy.

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CASE REPORT

A case of long-standing autoimmune type 1 diabetes with common variable immunodeficiency

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Abstract Several lines of evidence have suggested that pancreatic β-cell destruction is caused by inflammatory cellular responses mediated by T lymphocytes in individuals with type 1A diabetes. B lymphocytes, which play an important role in the production of autoantibodies to β-cell antigens such as insulin, glutamic acid decarboxylase (GAD) or insulinoma associated antigen 2 (IA-2) in type 1A diabetes, are also known as professional antigen-presenting cells and T-lymphocyte activators. Here, we report a case of long-standing autoimmune type 1 diabetes with common variable immunodeficiency, which is known as a functional deficiency of B lymphocytes. A 51-year-old man was admitted to our hospital because of hyperglycemia. He had suffered from frequent bacterial infections from early childhood. At 16 years old, he was diagnosed with common variable immunodeficiency. At age 27, he experienced sudden-onset diabetic ketosis and was diagnosed with type 1 diabetes. Enzyme-linked immunospot (ELISPOT) assay recently revealed that interferon-γ-producing T lymphocytes but not interleukin 4-producing T lymphocytes, which react with GAD and insulin $B_{1\text{-}18}$, were present at increased levels in his peripheral blood at 51 years old. This case represents the longest reported interval between onset of type 1 diabetes and confirmation of cell-mediated autoimmunity against pancreatic β-cells in a patient with common variable immunodeficiency.

Keywords IDDM · CVID · ELISPOT · GAD · IA-2

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Introduction

Several lines of evidence have suggested that pancreatic β-cell destruction is caused by inflammatory cellular responses mediated by T lymphocytes in individuals with type 1A diabetes [1, 2]. B lymphocytes, which play an important role in the production of autoantibodies to β cell antigens such as insulin, GAD or IA-2 in type 1A diabetes patients, are also known as professional antigen-presenting cells and T-lymphocyte activators.

Here, we report a case of long-standing autoimmune type 1 diabetes with common variable immunodeficiency (CVID), which is a functional deficiency of B lymphocytes.

Case report

In 2009, a 51-year-old man was admitted to our hospital because of hyperglycemia. He had suffered from frequent bacterial infections from early childhood. At 16 years old, he was diagnosed with CVID. At age 27, he had sudden-

onset diabetic ketosis and was diagnosed with type 1 diabetes. At admission to our hospital, his blood glucose level was 306 mg/dl, and urinary C peptide excretion was reduced to 8.7 μg/day. The serum IgG level was as low as 340 mg/dl (normal range 870–1,700 mg/dl), the IgA level was 57 mg/dl (normal range 110–410 mg/dl), and the IgM level was 31 mg/dl (normal range 35–220 mg/dl). Subcutaneous injection of insulin was started. He has been treated with monthly intravenous injections of γ-globulin (5,000 mg) since the age of 45. Immunoglobulin supplementation led to a marked reduction in the number of infections. Islet cell antibodies (ICA) and GAD antibodies were not detected either at 46 years old or on admission. IA-2 antibody, insulin autoantibodies (IAA), antinuclear antibody, thyroid-stimulating hormone (TSH) receptor antibody and thyroglobulin antibody tests were also negative.

Laboratory data on admission revealed normal blood count and blood chemistry. However, the CD19+ B-lymphocyte level (139/μl) but not the CD3+ T-lymphocyte level (1,160/μl) was decreased. The CD4/CD8 ratio had decreased to 30.5/46.0 (0.66). The HLA haplotypes of *DRB1-DQB1* were **04:05-*04:01* and **11:01-*03:01*. A glucagon tolerance test revealed the complete loss of endogenous insulin secretion capacity; the serum C-peptide concentrations before and 6 min after injection were undetectable (below 0.01 ng/ml).

We measured the responses of pancreatic β-cell-reactive peripheral T lymphocytes using an immunoglobulin-free enzyme-linked immunospot (ELISPOT) assay as described previously [3]. The mean number of interferon (IFN)-γ spots reactive to GAD_{65} and insulin B_{1-18} peptide was 7.5 and 3.5, respectively, in a duplicate assay. Interleukin (IL)-4 spots reactive to those peptides were not detected. Neither IFN-γ spots nor IL-4 spots reacted to insulin B_{9-23}, B_{10-24}, A_{1-15} and L_{7-23}. To compare the positivity among the other diabetic patients and control subjects in ELISPOT assay, the mean number of antigen-stimulated IFN-γ spots reactive to GAD_{65} was plotted after subtracting the background (T cells only). A significant IFN-γ response to the GAD_{65} peptide was observed in this patient (Fig. 1a). Data for other patients with type 1A diabetes and type 2 diabetes and for healthy controls were taken from our previous report [3].

Two-color flow cytometric analysis revealed decreased numbers of $CD10^-$ $CD19^+$ cells and $CD10^-$ $CD22^+$ cells (mature B lymphocytes) (Fig. 1b, c). However, the numbers of $CD10^+$ $CD19^-$ cells and $CD10^+$ $CD22^-$ cells (immature B lymphocytes) were not increased (Fig. 1b, c). The reference values of our methods were less than 1.0% for $CD10^+$ cells, 5.0–24.0% for $CD19^+$ cells and 2.0–17.0% for $CD22^+$ cells. $CD4^+$ $FoxP3^+$ regulatory T cells were 2.5% in this patient, while they were 3.6 (1.2–5.1)% [median (range)] in 20 healthy individuals [16].

Discussion

We report the first case of established T cell immunity in an autoimmune type 1 (type 1A) diabetes patient with CVID. Islet autoantibodies were not detected; however, an ELISPOT assay, a useful tool to detect T-lymphocyte-mediated autoimmunity directly with good reproducibility in type 1 diabetes patients [3, 4], revealed GAD- and insulin B_{1-18}-reactive Th1 cells, but not GAD- and insulin B_{1-18}-reactive Th2 cells among the peripheral lymphocytes in this patient. T-lymphocyte reactivity specific to beta cell

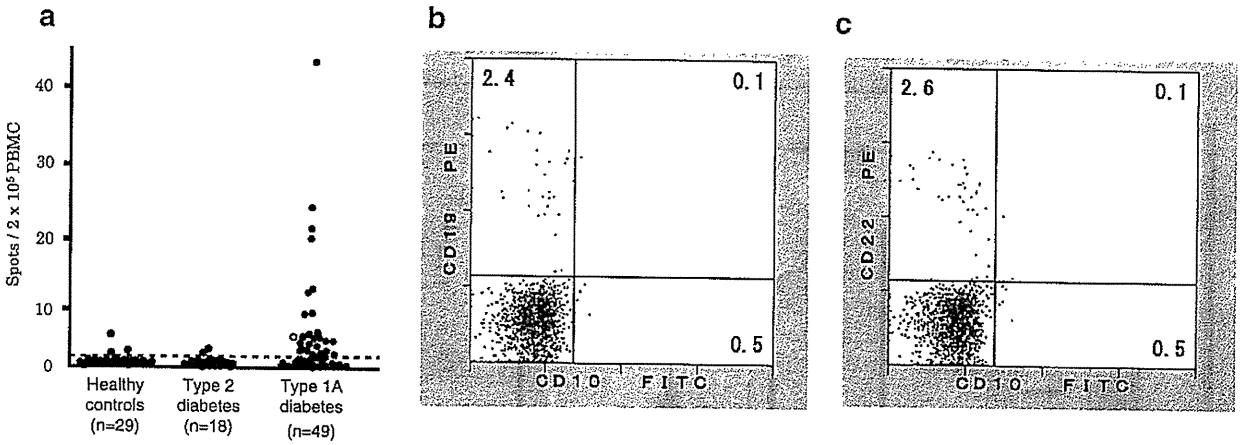

Fig. 1 a IFN-γ spots reactive to GAD_{65} in ELISPOT assays for subjects with type 1A diabetes or type 2 diabetes and for normal control subjects. The *open circle* represents our patient. Other data were taken from reference 3. Two-color flow cytometric analysis of our patient's PBMCs. A decreased number of $CD10^-$ $CD19^+$ cells and a normal number of $CD10^+$ $CD19^-$ cells (b) and a decreased number of $CD10^-$ $CD22^+$ cells and a normal number of $CD10^+$ $CD22^-$ cells (c) are shown

antigens of insulin B_{1-18} suggested the presence of a beta-cell-specific immune response. Higashide et al. [17] have reported that insulin B_{1-15} reactive Th1 cells are present in 6 of 18 recent-onset type 1 diabetic patients by ELISPOT assay, also suggesting the presence of insulin B_{1-18}-reactive Th1 cells in this patient indicates a long-lasting autoimmune response rather than acquired response induced by the long-lasting insulin treatment. There have already been several reports of probable type 1 diabetes with CVID, and ICA were detected in one of these patients. However, these patients did not have T lymphocyte immune reactivity to β cells at all [5–9].

CVID is a primary immune disorder characterized by hypogammaglobulinemia, antibody deficiency and recurrent infections [10]. This patient had (1) repeated infections in his childhood but not in his babyhood, (2) low levels of IgM, IgG and IgA in sera, (3) a normal number of T lymphocytes and (4) decreased but not absent B lymphocytes in his peripheral blood.

Our examination suggested that both the number and function of B lymphocytes were reduced in this patient. Laboratory data revealed a decreased $CD19^+$ B-lymphocyte level (139/μl). Flow cytometric analysis revealed no insufficient maturation of B lymphocytes in this patient. The functional deficiency of B lymphocytes was not directly observed; however, the history of repeated infections and improvement resulting from γ-globulin supplementation suggests non-specific functional loss of B lymphocytes. The lack of islet-related autoantibodies in spite of the positive reaction for his T lymphocytes against islet autoantigens might also indicate the reduction of B-lymphocyte function. On the other hand, the number of T lymphocyte including regulatory T cells, was normal in this patient.

This patient suffers from type 1A diabetes. This fact might indicate that B-lymphocyte insufficiency is not essential to the development of human type 1A diabetes despite the evidence in non-obese diabetic (NOD) mice, a rodent model [11–13]. The number of B lymphocytes in human insulitis lesions is low [2]. The effect of anti-CD20 therapy was limited to the patients with established autoimmune type 1 diabetes [14]. Type 1 diabetes has even been reported in a patient with X-linked severe agammaglobulinemia [15]. All of these findings suggest that B lymphocytes are not necessary to develop autoimmune β cell destruction in humans. Our present case with type 1A diabetes and CVID supports this concept for human type 1A diabetes. In addition, our patient had a positive reaction of T lymphocytes to islet autoantigens even 24 years after the onset of type 1 diabetes. These results might indicate that B-lymphocyte-mediated immunodeficiency was able to maintain anti-β cell autoimmunity long after disease onset.

Autoimmune diseases are more frequent in CVID patients than in general population [18, 19]. However, the prevalence of type 1 diabetes in CVID patients is not well documented. The established diagnosis of type 1 diabetes is sometimes difficult in CVID patients because islet autoantibodies are negative despite the presence of islet autoimmunity shown in the present case. It may underrepresent the prevalence of type 1 diabetes in CVID patients.

In conclusion, this case represents the longest reported interval between onset of type 1 diabetes and confirmation of cell-mediated autoimmunity against pancreatic β-cells in a patient with CVID.

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Class II HLA genotype in fulminant type 1 diabetes: A nationwide survey with reference to glutamic acid decarboxylase antibodies

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ABSTRACT

Aims/Introduction: Fulminant type 1 diabetes is a subtype of type 1 diabetes characterized by a remarkably abrupt onset of insulin-deficient hyperglycemia within a few days. The aim of the present study was to clarify characteristic class II HLA genotypes in a large number of patients with fulminant type 1 diabetes to date.

Materials and Methods: We analyzed the HLA-*DRB1* and *DQB1* genotypes, and their haplotypes in 207 patients with fulminant type 1 diabetes and 325 control subjects in the Japanese population.

Results: The frequencies of the *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* haplotypes were significantly higher, and those of the *DRB1*01:01-DQB1*05:01*, *DRB1*15:02-DQB1*06:01* and *DRB1*08:03-DQB1*06:01* haplotypes were significantly lower in patients with fulminant type 1 diabetes than in the control subjects. Combination analysis showed that the frequencies of homozygotes with *DRB1*04:05-DQB1*04:01* [odds ratio (OR) 7.0] and *DRB1*09:01-DQB1*03:03* (OR 9.5) were significantly higher in patients with fulminant type 1 diabetes. Within a limited portion of patients with fulminant type 1 diabetes with antibodies to glutamic acid decarboxylase (GADab; $n = 25$), the frequency of *DRB1*09:01-DQB1*03:03*, but not *DRB1*04:05-DQB1*04:01*, was significantly higher than in control subjects (44.0% vs 13.7%; $Pc < 0.05$, OR 5.0).

[Correction to last line of Results, added after online publication 29 July 2011: "OR 5.1" is changed to "OR 5.0".]

Conclusions: Our large-scale study showed the characteristic class II HLA genotypes in fulminant type 1 diabetes, and implicated that genetic contribution to disease susceptibility is distinct between GADab-positive and GADab-negative fulminant type 1 diabetes.
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KEY WORDS: Fulminant type 1 diabetes, HLA, Glutamic acid decarboxylase

INTRODUCTION

Fulminant type 1 diabetes is a novel subtype of type 1 diabetes identified in 2000[1–3]. It is defined as diabetes that results from the extremely rapid and almost entire destruction of pancreatic β-cells within a few days. The clinical characteristics of this subtype are different in many aspects from those of typical type 1A diabetes[3]. Although fulminant type 1 diabetes resembles the typical form of type 1 diabetes in that it is characterized by high plasma glucose levels accompanied by ketosis or ketoacidosis, it clearly differs by an extremely acute onset of diabetes, which is confirmed by nearly normal HbA_{1c} levels against high plasma glucose concentration, and virtually no C-peptide secretion at the onset of the disease, indicating that the process of pancreatic β-cell destruction is very rapid.

Fulminant type 1 diabetes is common in the Asian population; it accounts for approximately 20% of ketosis-onset type 1 diabetes in Japan[2,3] and 7% in Korea[4,5]. Furthermore, several cases have been reported from China[6], Taiwan[7], the Philippines[8], Malaysia[9] and France[10].

It is suggested that both genetic factors[11–13] and environmental factors, such as viral infection[14–19], contribute to the pathogenesis of this disease. In regard to genetic factors, it has been reported that class II HLA strongly confers susceptibility to the development of fulminant type 1 diabetes. In the analysis of the serological typing of class II HLA, we have shown that HLA-DR4-DQ4 was significantly more frequent in fulminant type 1 diabetes in Japan[12]. Several studies have so far reported the association of class II HLA genotype with fulminant type 1 diabetes[20–22]; however, the number of patients was limited in these reports as a result of the low incidence of type 1 diabetes in general, fulminant type 1 diabetes in particular, in the Japanese population.

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The aim of the present study was thus to investigate the class II HLA genotypes and re-evaluate the contribution of the class II HLA to susceptibility and resistance to fulminant type 1 diabetes in a large number of patients.

MATERIALS AND METHODS

Subjects and Methods

We examined 207 patients with fulminant type 1 diabetes and 325 healthy control subjects in Japan. Among them, 152 patients with fulminant type 1 diabetes were registered with the committee of the Japan Diabetes Society, and data for the other 55 patients were collected from reports in the literature from June 2000 to March 2007.

Inclusion criteria for fulminant type 1 diabetes were: (i) ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms; (ii) urinary C-peptide excretion <10 μg/day or fasting serum C-peptide <0.3 ng/mL (0.10 nmol/L) or serum C-peptide <0.5 ng/mL (0.17 nmol/L) after glucagon injection or meal load soon after disease onset; and (iii) plasma glucose level ≥16.0 mmol/L (288 mg/dL) and HbA_{1c} <8.9% at the first visit[2]. Healthy control subjects had normal glucose tolerance as assessed by a 75 g oral glucose tolerance test, had no family history of diabetes, and resided in the Ehime and Osaka areas as described previously[23]. GAD antibodies (GADab) were positive in 25 patients and negative in 182 patients (Table 1). We also analyzed 15 patients with pregnancy-associated fulminant type 1 diabetes (PF), 51 female patients of child-bearing age (13–49 years) with fulminant type 1 diabetes that was not associated with pregnancy (NPF) and 70 female control subjects of child-bearing age.

The present study was approved by the ethics committee of the Japan Diabetes Society, and informed consent was obtained from all subjects. The detailed characteristics of these subjects are shown in Table 1.

The value for HbA_{1c} (%) was estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[24].

Typing of HLA-DR and -DQ

HLA-DRB1 and *-DQB1* were genotyped by the PCR sequence-specific primer and PCR sequence-specific oligonucleotide methods (Invitrogen, Carlsbad, CA, USA). The most probable *DRB1-DQB1* haplotypes were deduced from known linkage disequilibria.

Statistical Analysis

Clinical data of GADab-negative and -positive fulminant type 1 diabetes was analyzed by using chi-squared-test or Kruskal–Wallis test. Allele frequencies were estimated by direct counting. Genotypes, whose total frequencies in both total subjects with fulminant type 1 diabetes and control subjects were five or more than five, were listed in the present study. The significance of the difference in distribution of alleles between patients with fulminant type 1 diabetes and healthy control subjects was determined by a chi-squared-test. *P*-values were corrected by using the number of different alleles tested (denoted as *Pc*). Statistical significance was defined as $Pc < 0.05$.

RESULTS

Characteristics of GADab-Negative and -Positive Fulminant Type 1 Diabetes

GADab was detected in 25 (12.1%) of 207 patients with fulminant type 1 diabetes in the present study. Therefore, first of all, we compared detailed characteristics between GADab-negative and -positive fulminant type 1 diabetes (Table 1). There were no differences between the two groups in age, body mass index, mean HbA_{1c} level at onset and presence or absence of family history of type 1 or type 2 diabetes in first-degree relatives. One, but not another, allele of class II HLA haplotype was common between two patients (father and his son) with a family history of

Table 1 | Clinical characteristics of patients with fulminant type 1 diabetes

	Total	With GADab	Without GADab	Control
n	207	25 (12.1)	182 (87.9)	325
Sex (male/female)	118/89 (57.0)	20/5 (80.0)	98/84 (53.8)	202/123 (62.2)
Pregnancy (PF*/NPF†)	15/51 (22.7)	0/5 (0.0)	15/49 (23.4)	ND
Age at disease onset (years)	41 (0–87)	43 (0–75)	41 (1–87)	47 (25–78)
Body mass index (kg/m^2)	21.1 ± 3.2‡	20.9 ± 3.4§	21.2 ± 3.2¶	ND
Family history of type 1 diabetes	5/157 (3.1)	0/20 (0.0)	5/137 (3.5)	0/0 (0.0)
Family history of type 2 diabetes	11/151 (6.8)	2/18 (10.0)	9/133 (6.3)	0/0 (0.0)
Family history of unclassified diabetes	6/156 (3.7)	1/19 (5.0)	5/137 (3.5)	0/0 (0.0)
HbA_{1c} at disease onset (%)	6.6 ± 0.8	6.7 ± 0.7	6.6 ± 0.8	ND

GADab, antibodies to glutamic acid decarboxylase; ND, not determined.
Data are *n*, median (range), mean ± SD, (±), or *n* (%).
*Pregnancy-associated fulminant type 1 diabetes; †Female patients of child-bearing age (13–49 years) with fulminant type 1 diabetes not associated with pregnancy; ‡except seven children; §Except two children; ¶Except five children.

fulminant type 1 diabetes. GADab was measured by radioimmunoassay[1], except for one patient in whom GADab was measured by radioligand binding assay[25]. There were no differences in sensitivity and specificity between the two assays. GADab was determined within a week after the onset of diabetes, except for two patients in each hospital. GADab was negative in one patient measured 6 months after the onset and positive in another patient measured 16 years after the onset. The median level of GADab was 3.0 U/mL (range 1.5–20.0 U/mL). In 78% of GADab-positive patients, the titer was <10 U/mL at the onset of disease and GADab became negative within 2 years during the follow up. Of 25 GADab-positive patients with fulminant type 1 diabetes, IA-2ab was negative in 16 patients and not measured in the other nine patients. In GADab-positive patients with fulminant type 1 diabetes, median duration of hyperglycemic symptoms was 4 days (range 0–11 days); median HbA_{1c} level was 6.7% (range 5.6–8.3%) despite very high plasma glucose levels (median 700, range 313–1944 mg/dL), showing the similarity in the clinical features, except the positivity of GADab, between GADab-positive and GADab-negative fulminant type 1 diabetes.

Table 2 | *DRB1* and *DQB1* alleles in patients with fulminant type 1 diabetes and control subjects

		Fulminant			Control (n = 650†)	Total vs control		GADab(+) vs control		GADab(–) vs control	
		Total (n = 414†)	GADab(+) (n = 50†)	GADab(–) (n = 364†)							
		n (%)	n (%)	n (%)	n (%)	Pc	OR	Pc	OR	Pc	OR
DRB1	*01:01	9 (2.2)	0 (0.0)	9 (2.5)	50 (7.7)	2.8×10^{-3}	0.27	NS		0.015	0.30
	*04:01	6 (1.4)	1 (2.0)	5 (1.4)	5 (0.8)	NS		NS		NS	
	*04:03	6 (1.4)	1 (2.0)	5 (1.4)	21 (3.2)	NS		NS		NS	
	*04:05	135 (32.6)	11 (22.0)	124 (34.0)	92 (14.2)	1.7×10^{-11}	2.9	NS		2.4×10^{-12}	3.1
	*04:06	3 (0.7)	1 (2.0)	2 (0.5)	23 (3.5)	NS		NS		NS	
	*04:07	1 (0.2)	0 (0.0)	1 (0.3)	5 (0.8)	NS		NS		NS	
	*04:10	13 (3.1)	0 (0.0)	13 (3.6)	9 (1.4)	NS		NS		NS	
	*08:02	14 (3.4)	2 (4.0)	12 (3.3)	30 (4.6)	NS		NS		NS	
	*08:03	13 (3.1)	1 (2.0)	12 (3.3)	58 (8.9)	5.0×10^{-3}	0.33	NS		0.015	0.35
	*09:01	106 (25.6)	22 (44.0)	84 (23.1)	90 (13.8)	3.1×10^{-5}	2.1	4.6×10^{-7}	4.9	4.1×10^{-3}	1.9
	*10:01	1 (0.2)	0 (0.0)	1 (0.3)	9 (1.4)	NS		NS		NS	
	*11:01	3 (0.7)	0 (0.0)	3 (0.8)	13 (2.0)	NS		NS		NS	
	*12:01	7 (1.7)	1 (2.0)	6 (1.6)	27 (4.2)	NS		NS		NS	
	*12:02	5 (1.2)	2 (4.0)	3 (0.8)	9 (1.4)	NS		NS		NS	
	*13:02	23 (5.6)	1 (2.0)	22 (6.0)	26 (4.0)	NS		NS		NS	
	*14:01	7 (1.7)	1 (2.0)	6 (1.6)	23 (3.5)	NS		NS		NS	
	*14:03	1 (0.2)	0 (0.0)	1 (0.3)	6 (0.9)	NS		NS		NS	
	*14:05	3 (0.7)	0 (0.0)	3 (0.8)	12 (1.8)	NS		NS		NS	
	*14:06	3 (0.7)	0 (0.0)	3 (0.8)	7 (1.1)	NS		NS		NS	
	*15:01	22 (5.3)	3 (6.0)	19 (5.2)	45 (6.9)	NS		NS		NS	
	*15:02	16 (3.9)	0 (0.0)	16 (4.4)	73 (11.2)	5.1×10^{-4}	0.32	NS		4.9×10^{-3}	0.36
	*16:02	8 (1.9)	2 (4.0)	6 (1.6)	6 (0.9)	NS		NS		NS	
	Others	9 (2.2)	1 (2.0)	8 (2.2)	11 (1.6)						
DQB1	*03:01	18 (4.3)	3 (6.0)	15 (4.1)	62 (9.5)	0.019	0.43	NS		0.020	0.41
	*03:02	21 (5.1)	2 (4.0)	19 (5.2)	67 (10.3)	0.028	0.46	NS		NS	
	*03:03	109 (26.3)	22 (44.0)	87 (23.9)	97 (14.9)	4.9×10^{-5}	2.0	1.5×10^{-6}	4.5	4.1×10^{-3}	1.8
	*04:01	133 (32.1)	11 (22.0)	122 (33.5)	91 (14.0)	1.7×10^{-11}	2.9	NS		2.8×10^{-12}	3.1
	*04:02	22 (5.3)	2 (4.0)	20 (5.5)	27 (4.2)	NS		NS		NS	
	*05:01	11 (2.7)	0 (0.0)	11 (3.0)	59 (9.1)	4.2×10^{-4}	0.27	NS		2.9×10^{-3}	0.31
	*05:02	11 (2.7)	3 (6.0)	8 (2.2)	19 (2.9)	NS		NS		NS	
	*05:03	8 (1.9)	1 (2.0)	7 (1.9)	23 (3.5)	NS		NS		NS	
	*06:01	30 (7.2)	1 (2.0)	29 (8.0)	132 (20.3)	8.1×10^{-8}	0.31	0.030	0.08	2.7×10^{-6}	0.34
	*06:02	21 (5.1)	3 (6.0)	18 (4.9)	44 (6.8)	NS		NS		NS	
	*06:04	20 (4.8)	1 (2.0)	19 (5.2)	26 (4.0)	NS		NS		NS	
	Others	10 (2.4)	1 (2.0)	9 (2.5)	4 (0.6)						

GADab, antibodies to glutamic acid decarboxylase; NS, not significant.
Pc-, *P*-values corrected for number of different alleles tested (×22 for *DRB1*and ×11 for *DQB1*).
†Allele number.

Frequencies of Alleles of HLA-DRB1 and DQB1

As shown in Table 2, the allele frequencies of *DRB1*04:05*, *DRB1*09:01*, *DQB1*04:01* and *DQB1*03:03* were significantly higher, and those of *DRB1*01:01*, *DRB1*08:03*, *DRB1*15:02*, *DQB1*03:01*, *DQB1*03:02*, *DQB1*05:01* and *DQB1*06:01* were significantly lower in total subjects with fulminant type 1 diabetes than in control subjects.

Similarly, the allele frequencies of *DRB1*04:05*, *DRB1*09:01*, *DQB1*04:01* and *DQB1*03:03* were significantly higher, and those of *DRB1*01:01*, *DRB1*08:03*, *DRB1*15:02*, *DQB1*03:01*, *DQB1*05:01* and *DQB1*06:01* were significantly lower in GADab-negative patients with fulminant type 1 diabetes than in control subjects.

In contrast, the allele frequencies of *DRB1*09:01* and *DQB1*03:03* were significantly higher, and that of *DQB1*06:01* was significantly lower in GADab-positive patients with fulminant type 1 diabetes than in control subjects (Table 2).

The frequencies of *DRB1*09:01* and *DQB1*03:03* were significantly higher in GADab-positive patients than in GADab-negative patients with fulminant type 1 diabetes (44.0 vs 23.1%, *Pc* = 0.033 and 44.0 vs 23.9%, *Pc* = 0.027, respectively).

Frequencies of the Genotypes of DRB1-DQB1 Haplotypes

As shown in Table 3, *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* are significantly more frequent in total subjects with fulminant type 1 diabetes than in controls. *DRB1*15:02-DQB1*06:01*, but not *DRB1*15:01-DQB1*06:02*, was significantly less frequent in these patients than in control subjects. Furthermore, *DRB1*01:01-DQB1*05:01* and *DRB1*08:03-DQB1*06:01* were significantly less frequent in these patients than in controls.

Similarly, the frequencies of *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* were significantly higher and those of *DRB1*01:01-DQB1*05:01*, *DRB1*15:02-DQB1*06:01* and *DRB1*08:03-DQB1*06:01* were significantly lower in

Table 3 | *DRB1-DQB1* haplotypes in patients with fulminant type 1 diabetes and control subjects

DRB1-DQB1	Fulminant			Control (n = 650†)	Total vs control		GADab(+) vs control		GADab(−) vs control	
	Total (n = 414†)	GADab(+) (n = 50†)	GADab(−) (n = 364†)							
	n (%)	n (%)	n (%)	n (%)	Pc	OR	Pc	OR	Pc	OR
**01:01-*05:01*	9 (2.2)	0 (0.0)	9 (2.5)	50 (7.7)	3.1×10^{-3}	0.27	NS	0.12	0.016	0.30
**04:01-*03:01*	3 (0.7)	1 (2.0)	2 (0.5)	5 (0.7)	NS		NS		NS	
**04:03-*03:02*	6 (1.4)	1 (2.0)	5 (1.4)	22 (3.2)	NS		NS		NS	
**04:05-*04:01*	135 (32.6)	11 (22.0)	124 (34.1)	92 (14.2)	2.0×10^{-11}	2.9	NS	1.7	2.7×10^{-12}	3.1
**04:06-*03:02*	3 (0.7)	1 (2.0)	2 (0.5)	23 (3.5)	NS		NS		NS	
**04:07-*03:02*	1 (0.2)	0 (0.0)	1 (0.3)	5 (0.7)	NS		NS		NS	
**04:10-*04:02*	13 (3.1)	0 (0.0)	13 (3.6)	9 (1.3)	NS		NS		NS	
**08:02-*03:02*	6 (1.4)	0 (0.0)	6 (1.6)	15 (2.2)	NS		NS		NS	
**08:02-*04:02*	8 (1.9)	2 (4.0)	6 (1.6)	16 (2.3)	NS		NS		NS	
**08:03-*06:01*	13 (3.1)	1 (2.0)	12 (3.3)	58 (8.9)	5.7×10^{-3}	0.33	NS	0.21	0.017	0.35
**09:01-*03:03*	105 (25.4)	22 (44.0)	83 (22.8)	89 (13.7)	3.8×10^{-5}	2.1	3.9×10^{-7}	5.0	5.2×10^{-3}	1.9
**10:01-*05:01*	1 (0.2)	0 (0.0)	1 (0.3)	10 (1.5)	NS		NS		NS	
**11:01-*03:01*	1 (0.2)	0 (0.0)	1 (0.3)	13 (1.9)	NS		NS		NS	
**12:01-*03:01*	4 (1.0)	1 (2.0)	3 (0.8)	21 (3.1)	NS		NS		NS	
**12:01-*03:03*	2 (0.5)	0 (0.0)	2 (0.5)	5 (0.7)	NS		NS		NS	
**12:02-*03:01*	4 (1.0)	1 (2.0)	3 (0.8)	9 (1.3)	NS		NS		NS	
**13:02-*06:04*	19 (4.6)	1 (2.0)	18 (4.9)	23 (3.5)	NS		NS		NS	
**14:01-*05:02*	2 (0.5)	0 (0.0)	2 (0.5)	13 (1.9)	NS		NS		NS	
**14:01-*05:03*	5 (1.2)	1 (2.0)	4 (1.1)	13 (1.9)	NS		NS		NS	
**14:03-*03:01*	1 (0.2)	0 (0.0)	1 (0.3)	6 (0.9)	NS		NS		NS	
**14:05-*05:03*	3 (0.7)	0 (0.0)	3 (0.8)	13 (1.9)	NS		NS		NS	
**14:06-*03:01*	3 (0.7)	0 (0.0)	3 (0.8)	7 (1.0)	NS		NS		NS	
**15:01-*06:02*	20 (4.8)	3 (6.0)	17 (4.7)	43 (6.6)	NS		NS		NS	
**15:02-*06:01*	16 (3.9)	0 (0.0)	16 (4.4)	79 (11.2)	9.5×10^{-5}	0.29	NS	0.07	1.2×10^{-3}	0.33
**16:02-*05:02*	7 (1.7)	2 (4.0)	5 (1.4)	6 (0.9)	NS		NS		NS	
Others	24 (5.8)	2 (4.0)	22 (6.0)	20 (2.9)						

GADab, antibodies to glutamic acid decarboxylase; NS, not significant.
Pc-, *P*-values corrected for number of different haplotypes tested (×25).
†Allele number.

GADab-negative patients with fulminant type 1 diabetes than in control subjects.

In contrast, only *DRB1*09:01-DQB1*03:03* was significantly more frequent in GADab-positive patients with fulminant type 1 diabetes than in controls. The frequency of *DRB1*09:01-DQB1*03:03* was significantly higher (44.0 vs 22.8%, *Pc* = 0.031) in GADab-positive patients than in GADab-negative patients with fulminant type 1 diabetes.

Comparison between DRB1*04:05-DQB1*04:01 and DRB1*09:01-DQB1*03:03 Haplotypes

To clarify the difference in the genetic contribution of the two major HLA haplotypes, *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03*, to fulminant type 1 diabetes, we analyzed the frequencies of homozygotes and heterozygotes with *DRB1*04:05-DQB1*04:01* and/or *DRB1*09:01-DQB1*03:03* in patients with this form of diabetes and control subjects. As shown in Table 4, homozygotes with both *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03* were significantly more frequent in total subjects of fulminant type 1 diabetes than in control subjects. Heterozygotes with *DRB1*04:05-DQB1*04:01*, but not *DRB1*09:01-DQB1*03:03*, were also significantly more frequent in these patients than in control subjects.

Similarly, both homozygotes and heterozygotes with *DRB1*04:05-DQB1*04:01* were significantly more frequent in GADab-negative patients with fulminant type 1 diabetes than in control subjects. Homozygotes, but not heterozygotes, with *DRB1*09:01-DQB1*03:03* were present significantly more frequently in GADab-negative patients than in control subjects.

In contrast, both homozygotes and heterozygotes with *DRB1*09:01-DQB1*03:03* were significantly more frequent in GADab-positive patients with fulminant type 1 diabetes than in control subjects. Furthermore, neither homozygotes nor heterozygotes with *DRB1*04:05-DQB1*04:01* were associated with GADab-positive patients with fulminant type 1 diabetes.

When analyzed by using a 2 × 3 contingency table (homozygote, heterozygote and null of *DRB1*04:05-DQB1*04:01* or *DRB1*09:01-DQB1*03:03* between GADab-positive and GADab-negative patients; Table 4), there was a significant difference in the frequency of *DRB1*09:01-DQB1*03:03* ($P = 0.0093$), but not in the frequency of *DRB1*04:05-DQB1*04:01* ($P = 0.29$), between GADab-positive and GADab-negative patients.

To further investigate the disease susceptibility and protection provided by HLA haplotypes in fulminant type 1 diabetes, we examined the genotypic combinations classified as high-frequency haplotypes (*DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03*) and low-frequency haplotypes (*DRB1*01:01-DQB1*05:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*15:02-DQB1*06:01*) in patients with fulminant type 1 diabetes and in control subjects. As shown in Table 5, none of low-frequency haplotypes, such as *DRB1*01:01-DQB1*05:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*15:02-DQB1*06:01*, conferred protection to fulminant type 1 diabetes in combination with high-frequency haplotypes, such as *DRB1*04:05-DQB1*04:01* and *DRB1*09:01-DQB1*03:03*, although the number of patients was small.

Frequencies of the Genotypes of DRB1-DQB1 Haplotypes in Pregnancy

*DRB1*04:05-DQB1*04:01* was found to be significantly more frequent in the NPF group than in control subjects, whereas *DRB1*09:01-DQB1*03:03* was not significantly more frequent in either PF or NPF group compared with the controls (Table S1).

Homozygotes with *DRB1*04:05-DQB1*04:01* were significantly more frequent in the NPF group than in control subjects (Table S2). The frequency of homozygotes with *DRB1*04:05-DQB1*04:01* tended to be lower in the PF group than in the NPF group, but there was no significant difference between the groups. In contrast, neither homozygotes nor heterozygotes with *DRB1*09:01-DQB1*03:03* were associated with either the PF or NPF groups compared with the controls.

Table 4 | Combination of HLA-*DRB1-DQB1* haplotype in patients with fulminant type 1 diabetes and control subjects

DRB1-DQB1/DRB1-DQB1	Fulminant			Control ($n = 325$)	Total vs control		GADab(+) vs control		GADab(–) vs control	
	Total ($n = 207$)	GADab(+) ($n = 25$)	GADab(–) ($n = 182$)							
	n (%)	*n* (%)	*n* (%)	*n* (%)	*Pc*	OR	*Pc*	OR	*Pc*	OR
**04:05-*04:01/*04:05-*04:01*	31 (15.0)	2 (8.0)	29 (15.9)	8 (2.5)	2.0×10^{-7}	7.0	NS	3.4	6.6×10^{-8}	7.5
**04:05-*04:01/X*	73 (35.3)	7 (28.0)	66 (36.3)	76 (23.4)	8.8×10^{-3}	1.8	NS	1.3	5.8×10^{-3}	1.9
X/X	103 (49.8)	16 (64.0)	87 (47.8)	241 (74.2)	2.9×10^{-8}	0.35	NS	0.62	7.8×10^{-9}	0.32
**09:01-*03:03 *09:01-*03:03*	22 (10.6)	5 (20.0)	17 (9.3)	4 (1.2)	8.0×10^{-6}	9.5	1.3×10^{-6}	20.1	9.4×10^{-5}	8.3
**09:01-*03:03/Y*	61 (29.5)	12 (48.0)	49 (26.9)	81 (24.9)	NS	1.3	0.035	2.8	NS	1.1
Y/Y	124 (59.9)	8 (32.0)	116 (63.7)	240 (73.8)	2.2×10^{-3}	0.53	2.8×10^{-5}	0.17	NS	0.62

NS, not significant; GADab, antibodies to glutamic acid decarboxylase.
Pc-, *P*-values corrected for number of different haplotypes tested. *X* does not contain *DRB1*04:05-DQB1*04:01*. *Y* does not contain *DRB1*09:01-DQB1*03:03*.

Table 5 | Genotypic combination of *DRB1-DQB1* haplotype in patients with fulminant type 1 diabetes and control subjects

Allele frequency High/Low *DRB1-DQB1/ DRB1-DQB1*	Fulminant			Control (n = 325)	Total vs control		GADab(+) vs control		GADab(−) vs control	
	Total (n = 207)	GADab(+) (n = 25)	GADab(−) (n = 182)							
	n (%)	n (%)	n (%)	n (%)	P	OR	P	OR	P	OR
**04:05-*04:01/*										
**01:01-*05:01*	2 (1.0)	0 (0.0)	2 (1.1)	10 (2.9)	NS	0.31	NS	0.59	NS	0.35
**08:03-*06:01*	4 (1.9)	0 (0.0)	4 (2.2)	4 (1.2)	NS	1.6	NS	1.4	NS	1.8
**15:02-*06:01*	5 (2.4)	0 (0.0)	5 (2.7)	9 (2.6)	NS	0.87	NS	0.65	NS	0.99
**09:01-*03:03/*										
**01:01-*05:01*	1 (0.5)	0 (0.0)	1 (0.5)	7 (2.0)	NS	0.22	NS	0.83	NS	0.25
**08:03-*06:01*	4 (1.9)	0 (0.0)	4 (2.2)	7 (2.0)	NS	0.90	NS	0.83	NS	1.0
**15:02-*06:01*	2 (1.0)	0 (0.0)	2 (1.1)	15 (4.6)	0.037	0.20	NS	0.39	NS	0.23

GADab, antibodies to glutamic acid decarboxylase; NS, not significant.

DISCUSSION

The two important findings obtained from the present study were as follows: (i) the contribution of HLA genes to fulminant type 1 diabetes was clearly shown in a large-scale study; and (ii) the contribution of HLA genes to fulminant type 1 diabetes was different between GADab-positive and GADab-negative patients.

First, the present large-scale study has clarified the contribution of HLA genes to fulminant type 1 diabetes. We have reconfirmed that *DRB1*04:05-DQB1*04:01*, but not *DRB1*04:10-DQB1*04:02*, which also encodes DR4-DQ4, confers a strong predisposition to fulminant type 1 diabetes. Analysis of the combination of the HLA-*DRB1-DQB1* haplotype has shown that both homozygotes and heterozygotes with *DRB1*04:05-DQB1*04:01* show a strong effect regarding predisposition to fulminant type 1 diabetes (OR 7.0 and 1.8, respectively), as shown in a previous nationwide multicenter study[12,13]. [Correction to previous sentence, added after online publication 29 July 2011: "OR 6.4 and 1.9" is changed to "OR 7.0 and 1.8".] These findings suggest that *DRB1*04:05-DQB1*04:01* plays an important role in the development of fulminant type 1 diabetes.

We have also shown that the *DRB1*01:01-DQB1*05:01*, *DRB1*08:03-DQB1*06:01* and *DRB1*15:02-DQB1*06:01* haplotypes are negatively associated with fulminant type 1 diabetes. It is well known that both haplotypes of *DRB1*15:02-DQB1*06:01* and *DRB1*15:01-DQB1*06:02* encode DR2-DQ1[26]. In a previous study, we analyzed the serological subtype of HLA-DR-DQ and showed that the frequency of DR2-DQ1 was significantly lower in fulminant type 1 diabetes than in the control[12]. The present study has shown that *DRB1*15:02-DQB1*06:01*, but not *DRB1*15:01-DQB1*06:02*, which encode DR2-DQ1, was negatively associated with fulminant type 1 diabetes. Regarding the combination analysis, in the Japanese population, protective haplotypes, such as *DRB1*15:01-DQB1*06:02* and *DRB1*15:02-DQB1*06:01*, provide strong protection against type 1A diabetes regardless of the presence of susceptible haplotypes, such as *DRB1*09:01-DQB1*03:03* and *DRB1*04:05-DQB1*04:01*[13,26–31]. However, no such protective effect was observed in fulminant type 1 diabetes. This might show that protective haplotypes are not superior to susceptible haplotypes in fulminant type 1 diabetes.

*DRB1*09:01-DQB1*03:03*, in addition to *DRB1*04:05-DQB1*04:01*, haplotype was positively associated with fulminant type 1 diabetes. Recently, we have reported the differences in the contribution of HLA to genetic susceptibility to three subtypes of Japanese type 1 diabetes, acute-onset, fulminant and slowly-progressive, and that *DRB1*04:05-DQB1*04:01*, but not *DRB1*09:01-DQB1*03:03*, was associated with fulminant type 1 diabetes[13]. However, *DRB1*09:01-DQB1*03:03* was also high in frequency in the present study. We have two hypotheses to explain this discrepancy. One is that the maximum number of samples in the present study enabled us to re-evaluate the association of class II HLA genotype with fulminant type 1 diabetes. Another is the high frequency of *DRB1*09:01-DQB1*03:03* haplotype in GADab-positive patients with fulminant type 1 diabetes included in the present study. *DRB1*09:01-DQB1*03:03* conferred strong susceptibility to GADab-positive fulminant type 1 diabetes (OR 5.0). In addition, it has been reported that *DRB1*09:01-DQB1*03:03*, but not *DRB1*04:05-DQB1*04:01*, confers strong susceptibility to the disease development in pregnancy-associated fulminant type 1 diabetes in Japanese[32]. A similar trend was also observed in the present study, although the difference was not significant.

Second, the present study has clarified that the contribution of HLA genes to fulminant type 1 diabetes was different between GADab-positive and GADab-negative patients despite the similar clinical status. In the present large-scale study, the majority of fulminant type 1 diabetes, GADab-negative patients, was characterized by the predominance of *DRB1*04:05-DQB1*04:01* both in homozygous and heterozygous states. In contrast, *DRB1*09:01-DQB1*03:03*, but not *DRB1*04:05-DQB1*04:01*, was predominant in GADab-positive patients with fulminant type 1 diabetes. In addition, the protective effect of the *DRB1*15:02-DQB1*06:01* haplotype tended to be stronger in GADab-positive (0.0%, OR 0.07) than in GADab-negative

fulminant type 1 diabetes (4.4%, OR 0.33). In contrast, it is well known that the *DRB1*09:01-DQB1*03:03* haplotype is frequent in GADab-positive or typical autoimmune diabetic patients in Japan[13,27,28,31]. Kawabata *et al.* showed that the *DRB1*09:01-DQB1*03:03* haplotype confers much stronger susceptibility to Japanese typical autoimmune type 1 diabetes when present in a homozygous state and that the *DRB1*09:01-DQB1*03:03* haplotype predisposes in a recessive fashion. *DRB1*15:02-DQB1*06:01* also shows strong protection to classical type 1A diabetes[30]. High frequency of *DRB1*09:01-DQB1*03:03* homozygous state was also observed in GADab-positive fulminant type 1 diabetes in the present study (OR 20.1). Taken together, these findings suggest the similarity in underlying genetic backgrounds between classical autoimmune type 1 diabetes and GADab-positive fulminant type 1 diabetes, but not GADab-negative fulminant type 1 diabetes.

In conclusion, the present large-scale study showed the characteristic class II HLA genotypes in fulminant type 1 diabetes. The present study also implied that genetic contribution to disease susceptibility is distinct between GADab-positive and GADab-negative fulminant type 1 diabetes. Consequently, this disorder might be heterogeneous, as reflected by class II HLA and GADab, and further divided into at least two subtypes.

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APPENDIX

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | *DRB1-DQB1* haplotypes in female patients with PF and NPF and in control subjects
Table S2 | Combination of HLA-*DRB1-DQB1* haplotype in female patients with PF and NPF and in control subjects

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Low CTLA-4 expression in CD4$^+$ helper T-cells in patients with fulminant type 1 diabetes

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ABSTRACT

Fulminant type 1 diabetes is a novel subtype of type 1 diabetes characterized by a remarkably abrupt onset of insulin-deficient hyperglycemia. An accelerated immune reaction has been suggested as the cause of markedly rapid beta cell loss in this disease, but the precise mechanism has not been clarified. We analyzed the expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) in CD4$^+$ helper T-cells in 16 patients with fulminant type 1 diabetes, 14 patients with type 1A diabetes, 10 patients with type 2 diabetes and 20 normal control subjects. There was a significant reduction in CTLA-4 expression in CD4$^+$ helper T-cells from patients with fulminant type 1 diabetes ($P<0.05$) compared with the other three groups. Low CTLA-4 expression was also observed in both CD4$^+$CD25^{high} T-cells and CD4$^+$CD25$^-$ T-cells. There was a significant negative correlation between the proliferation of CD4$^+$CD25$^-$ T-cells and the levels of CTLA-4. Intracellular expression of CTLA-4 in CD4$^+$ helper T-cells was not correlated with two *CTLA-4* polymorphisms. In conclusion, the expression of CTLA-4 in CD4$^+$ helper T-cells was low in patients with fulminant type 1 diabetes.

1. Introduction

Fulminant type 1 diabetes is a novel subtype of type 1 diabetes characterized by almost complete insulin deficiency resulting from the destruction of pancreatic beta cells [1,2]. The clinical characteristics of this disease are as follows: (1) remarkably abrupt onset; (2) very short duration of diabetic symptoms; (3) acidosis at the time of diagnosis; (4) negative findings in general for islet-related autoantibodies, such as anti-GAD antibodies (GAD Ab), insulin autoantibodies and anti-insulinoma-associated antigen 2 antibodies (IA-2 Ab); (5) virtually no C-peptide secretion; and (6) elevated serum pancreatic enzyme levels [1–4]. Of these, the abrupt onset and very short duration suggest that rapid loss of beta cells occurs in fulminant type 1 diabetes. In addition, massive cellular infiltration of T-cells and macrophages has been detected in the pancreas just after disease onset [5,6]. Increased T-cell responses against pancreatic beta cell antigens as detected by enzyme-linked immunospot (ELISPOT) assay have been proposed [7]. Based on these findings, we hypothesize that pancreatic beta cells could be destroyed by an accelerated immune response followed by T-cell activation, leading to the development of fulminant type 1 diabetes.

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an inhibitory immunoregulatory molecule that has an important role in adjusting the threshold for T-cell activation and preventing autoimmunity. Usually, CTLA-4 is only present on the surface of activated T-cells at low levels; the majority of CTLA-4 proteins is instead localized in intracellular compartments and is transported to the cell surface only in response to T-cell receptor ligation [8,9]. CTLA-4 function is mediated via both cell-extrinsic and cell-intrinsic mechanisms [10,11]. One cell-extrinsic mechanism is that regulatory T-cells (Tregs) suppress the proliferation of effector T-cells via the CTLA-4/B7 receptor/ligand system on antigen presenting cells (APCs). Phenotypic characterization of Tregs indicates that they are CD4$^+$ T cells that co-express constitutively high levels of interleukin (IL)-2R alpha (CD25) and the transcription factor forkhead box P3 (FOXP3) in addition to CTLA-4 [12–15]. In a murine study, Treg-specific CTLA-4 deficiency impaired the suppressive function of Tregs resulting in fatal systemic T-cell lymphoproliferation; in particular, Tregs mediated the down-regulation of CD80 and CD86 expression on APCs [16]. A cell-intrinsic mechanism that regulates CTLA-4 function is mediated by binding to the intracellular phosphatases (PTPases) SRC homology 2-domain-containing PTPase 2 and protein phosphatase 2A, and these PTPases could act to inhibit the function of T cell receptor (TCR) signaling tar-

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gets through dephosphorylation [10]. Indeed, CTLA-4 regulates the activation of effector T-cells independently of Tregs, as CTLA-4 expression in effector T cells in mice with a Treg-specific CTLA-4 deficiency can substantially delay the death of *CTLA-4*$^{-/-}$ mice [16].

These findings prompted us to assess the expression of CTLA-4 in CD4$^+$ helper T-cells (CD4$^+$CD25^{high} T-cells and CD4$^+$CD25$^-$ T-cells), the frequency of CD3$^+$FOXP3$^+$ T-cells and the function of CD4$^+$CD25^{high} T-cells for their role in accelerated beta cell death in patients with fulminant type 1 diabetes.

2. Materials and methods

2.1. Research design

We studied 16 patients with fulminant type 1 diabetes (Table 1) diagnosed according to the following inclusion criteria proposed by the Committee of the Japan Diabetes Society: (1) occurrence of diabetic ketosis or ketoacidosis soon (around 7 days) after the onset of hyperglycemic symptoms (elevation of urinary and/or serum ketone bodies at first visit); (2) plasma glucose level ≥16.0 mmol/l (≥288 mg/dl) and glycated hemoglobin (HbA1c) level <8.9% at first visit; (3) urinary C-peptide excretion <10 μg/day or fasting serum C-peptide level <0.3 ng/ml and <0.5 ng/ml (<0.17 nmol/l) after intravenous glucagon load (or after a meal) at onset [3]. We also performed a longitudinal analysis in two patients with recent-onset and two patients with long-standing fulminant type 1 diabetes. The value for HbA1c (%) is estimated as an NGSP equivalent value (%) calculated by the formula HbA1c (%) = HbA1c (JDS) (%) + 0.4%, based on the relational expression of HbA1c (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA1c (NGSP) [17].

As a control, we also studied 14 patients with long-standing type 1A diabetes, 10 patients with type 2 diabetes and 20 age-matched healthy control subjects (Table 1). The inclusion criteria for type 1A diabetes were as follows; (1) the presence of ketoacidosis at the onset of diabetes; (2) duration of hyperglycemic symptoms before insulin therapy of <3 months; (3) insulin replacement therapy required throughout the study period; and (4) the presence of GADAb or IA-2Ab.

This study was approved by the ethics committee of Osaka Medical College. Written informed consent was obtained from all patients.

2.2. Flow cytometric analyses

Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by density gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway), and immediately subjected to cellular staining. A total of 1×10^6 PBMCs was aliquoted (per tube) along with 20 μl of each appropriate test antibody or the respective isotype control. The cells were stained with the following antibodies: fluorescein isothiocyanate (FITC) anti-CD3 (clone HIT3a), phycoerythrin (PE) anti-CD25 (clone M-A251), PE anti-CD127 (clone hIL-7R-M21), allophycocyanin (APC)-CD152 (clone BNI3), APC-Cy^{TM}7-anti-CD25 (clone M-A251), peridinin chlorophyll protein (Per CP) anti-CD4 (clone SK3) (BD Bioscience, San Jose CA) and APC-FOXP3 (clone PCH101) (eBioscience, San Diego, CA). The following isotype control antibodies were used: FITC mouse IgG1 (40×), PerCP mouse IgG1 (MOPC-21), PE mouse IgG1 (MOPC-21), and APC-Cy^{TM}7 mouse IgG1 (MOPC-21) (BD Bioscience), and APC rat IgG2a (eBR2a) (eBioscience, San Diego, CA). After surface staining for 30 min (4 °C) in the dark, the cells were washed twice with cold PBS. Surface-stained cells were then permeabilized for intracellular CTLA-4 or FOXP3 staining using the Cytofix/Cytoperm^{TM} Fixation Permeabilization kit (BD Bioscience) or anti-human FOXP3 staining kit (eBioscience) according to the manufacturer's recommendations. Stained cells were then analyzed by flow cytometry using a BD FACSAria^{TM} Cell Sorter (BD Bioscience). BD FACSDiva Software was used for analysis of the cytometric data. At least 100,000 events were acquired from each sample.

2.3. Isolation of RNA and quantitative real-time polymerase chain reaction

CD4$^+$ T-cells were purified from PBMC by magnetic bead isolation (BD Bioscience). Total RNA was extracted from CD4$^+$ T-cells by using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostic GMBH, Mannheim, Germany). The mRNA levels were quantified using the TaqMan real-time RT-PCR method (LyghtCycler, Roche). The sequences of the primers and Roche universal probe number (#) were as follows: CTLA-4 (GenBank Accession No. L15006. 1: left primer tcacagctttctttgagca, right primer aggctgaaattgctttcaca, and #21 probe; GAPDH (NM002046): left primer agccacatcgctcagacac, right primer gcccaatacgaccaaatcc, and #60 probe.

2.4. Genotyping of CTLA-4 polymorphism

Two single nucleotide polymorphisms (SNPs) in the *CTLA-4* gene, +49G>A (rs231775) and CT60 (rs3087243), were genotyped in patients with fulminant type 1 diabetes, type 1A diabetes and normal control subjects as reported previously [18,19].

2.5. Cell isolation

PBMCs were stained with Per CP anti-CD4 (SK3) and PE anti-CD25 (M-A251). CD4$^+$CD25^{high} (CD25$^+$ top1% of CD3$^+$ T-cells) and CD4$^+$CD25$^-$ fractions were sorted with a BD FACSAria^{TM} Cell Sorter. The sorted CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ T-cell samples had purities greater than 95%. T-cell-depleted accessory cells were isolated by negative selection from PBMCs using anti-CD3 microbeads (BD Bioscience) according to the manufacturer's recommendations; these cells were by irradiated at 3000 rad. T-cell-depleted accessory cells were more than 98% pure.

2.6. Cell culture

Cells were cultured in RPMI 1640 medium (GIBCO) supplemented with penicillin (50 μg/ml)/streptomycin (50 μg/ml) (GIBCO) and 5% human type AB serum (Gemini Bio-Products) in U-bottom 96-well plates (Becton Dickinson France S.A.).

2.7. Proliferation assay

Proliferation assays were performed by culturing sorted CD4$^+$CD25$^-$ T-cells (1×10^4/well) with CD4$^+$CD25^{high} T-cells at various ratios (0:1, 1:0, 1:1/2, and 1:1/4) in the presence of 5×10^4 irradiated accessory cells. These co-cultures were stimulated using a combination of 5 μg soluble anti-CD3 (clone HIT3a) and 5 μg soluble anti-CD28 (clone CD28.2; BD Bioscience). All culture conditions were tested in triplicate. On day 5 of the culture, 1 μ Ci [^{3}H]thymidine (MP Biomedicals, Inc.) was added for the final 16 h of culture to assess proliferation. The percentage suppression was calculated as 100 − (mean cpm of co-cultures − CD4$^+$CD25^{high} T-cells alone/mean cpm of CD4$^+$CD25$^-$ T-cells alone) × 100 at a ratio of 1:1 (CD4$^+$CD25$^-$ T-cells: CD4$^+$CD25^{high} T-cells).

Table 1
Patients demographics.

	n	Gender (m/f)	Age (years)	GAD/IA-2 antibody positive (%)	*DRB1*0405*	*DRB1*0901*	Duration (years)
Fulminant type 1 diabetes	16	7/9	45 (22–67)	0/0	6/10	2/10	3.7 (0.04–10.3)
Type 1A diabetes	14	4/10	49 (25–66)	100/60($n=5$)	8/12	3/12	7.3 (2.0–23.3)
Type 2 diabetes	10	7/3	60 (32–74)	0/ND	2/7	1/7	8.0 (0.3–24.0)
Control subjects	20	14/6	43 (27–61)	ND/ND	2/13	1/13	

Data of *DRB1*0405* and *DRB1*0901* indicate number of patients with at least one allele/total number of patients; ND, not determined.

2.8. Cytokine determination

We measured serum cytokine concentrations in 8 patients with fulminant type 1 diabetes at the time of the quantitative analysis. Cytokine levels of IL-2, IL-4, IL-6, IL-10, IL-1beta, IL-17, gamma-interferon (IFN-γ), tumor necrosis factor (TNF)-α, and C-reactive protein (CRP) were measured by enzyme immunoassay or chemiluminescent enzyme immunoassay.

2.9. Statistical analysis

Differences between the groups were analyzed for statistical significance by the two-tailed unpaired Student's *t* test. We performed a power analysis to determine minimum number of samples required for each comparison. Power analysis was as follows: power.*t*.test (delta = 4, SD = 3, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 9.889068, power.*t*.test (delta = 3, SD = 2, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 8.06031, power.*t*.test (delta = 15, SD = 8, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 5.609417 and power.*t*.test (delta = 11, SD = 5, sig. level = 0.05, power = 0.8, *n* = NULL): *n* = 4.44319. A Spearman correlation was used to calculate the *r* value. $P<0.05$ was considered significant.

3. Results

3.1. Intracellular CTLA-4 and CTLA-4 mRNA expression in $CD4^+$ helper T-cells

To measure the levels of intracellular CTLA-4 expression in $CD4^+$ helper T-cells, we counted $CD4^+$ T-cells that co-expressed intracellular CTLA-4 (representative plots in Fig. 1A–C). There was a significant reduction in CTLA-4 expression in $CD4^+$ helper T-cells from patients with fulminant type 1 diabetes [median 6.8%, range 0.7–11.3%] compared with normal control subjects [median 13.5%, range 6.8–23.3%; $P=0.0002$], patients with type 2 diabetes [median 9.0%, range 7.3–17.4%; $P=0.0092$] and patients with type 1A diabetes [median 10.6%, range 4.6–18.8%; $P=0.0097$] (Fig. 2A). The levels of intracellular CTLA-4 expression in $CD4^+$ helper T-cells had no correlation to their disease duration either within any of the groups or when all patients were considered together. The expression of CTLA-4 mRNA in $CD4^+$ helper T-cells was not significantly different among the patients with fulminant type 1 diabetes [median 0.853, range 0.389–24.2: $n=7$], the group with type 1A diabetes [median 1.28, range 0.283–21.0: $n=7$], the group with type 2 diabetes [median 0.489, range 0.133–5.58: $n=4$] and the normal control subjects [median 1.25, range 0.671–10.9: $n=10$] (shown in arbitrary units).

3.2. Intracellular CTLA-4 expression in $CD4^+CD25^-$ T-cells

To determine the levels of intracellular CTLA-4 expression in $CD4^+CD25^-$ T-cells, we counted $CD4^+CD25^-$ T-cells that co-expressed intracellular CTLA-4. There was a significant reduction in CTLA-4 expression in $CD4^+CD25^-$ T-cells from patients with fulminant type 1 diabetes [median 4.0%, range 1.5–7.6%] compared with normal control subjects [median 9.7%, range 5.1–18.5%; $P=0.0003$], patients with type 2 diabetes [median 6.0%, range 4.5–13.7%; $P=0.0126$] and patients with type 1A diabetes [median 7.1%, range 5.0–12.5%; $P=0.0019$] (Fig. 2B). The levels of intracellular CTLA-4 expression in $CD4^+CD25^-$ T-cells had no correlation to their disease duration either within any of the groups or when all patients were considered together.

3.3. Intracellular CTLA-4 expression in $CD4^+CD25^{high}$ T-cells

To determine the levels of intracellular CTLA-4 expression in $CD4^+CD25^{high}$ T-cells, we counted the $CD4^+CD25^{high}$ T-cells that co-expressed intracellular CTLA-4. There was a significant reduction in CTLA-4 expression in $CD4^+CD25^{high}$ T-cells ($CD25^+$ top 1% of $CD3^+$ T-cells) from patients with fulminant type 1 diabetes [median 62.4%, range 45.2–76.6%] compared with normal control subjects [median 86.1%, range 69.3–93.1%; $P<0.0001$], patients with type 2 diabetes [median 81.4%, range 67.5–89.5%; $P<0.0001$] and patients with type 1A diabetes [median 75.9%, range 57.6–94.4%; $P=0.0020$] (Fig. 2C). Reduced intracellular CTLA-4 expression was also confirmed when we counted both $CD4^+CD25^{high}$ T-cells ($CD25^+$ top 2% of $CD3^+$ T-cells) and $CD4^+CD25^+CD127^{low}$ T-cells co-expressing intracellular CTLA-4 (Figs. 1B and 2D). The levels of intracellular CTLA-4 expression in $CD4^+CD25^{high}$ T-cells had no correlation to their disease duration either within any of the groups or when all patients were considered together.

3.4. Frequency of $CD3^+FOXP3^+$ T-cells and cytokine levels

To determine the frequency of Tregs, we counted the $CD3^+$ T-cells that co-expressed intracellular FOXP3 (representative plots in Fig. S1). The frequency of $CD3^+FOXP3^+$ T-cells did not differ among patients with fulminant type 1 diabetes [median 3.51%, range 1.00–6.17%], patients with type 1A diabetes [median 3.51%, range 1.87–7.40%], and normal controls [median 3.58%, range 1.16–5.11%] (Fig. 3). The frequency of $CD3^+FOXP3^+$ T-cells in patients with type 1A and fulminant type 1 diabetes had no correlation to their age, gender, disease duration, HbA1c level, C-peptide level or insulin dose. No significant alterations were observed in patients with recent-onset or established fulminant type 1 diabetes over a period of 6 months (Fig. S2). Cytokine levels in the fulminant type 1 diabetic patients had no correlation to the frequency of $CD3^+FOXP3^+$ T-cells (Table S1).

3.5. Function of $CD4^+CD25^{high}$ T-cells assessed by their ability to suppress the proliferation of $CD4^+CD25^-$ T-cells

When we co-cultured $CD4^+CD25^-$ T-cells with $CD4^+CD25^{high}$ T-cells at various ratios (0:1, 1:0, 1:1/2, and 1:1/4), cellular proliferation was similarly suppressed in a dose-dependent manner by $CD4^+CD25^{high}$ T-cells from patients with fulminant type 1 diabetes, patients with type 1A diabetes and normal controls (Fig. 4A). No significant differences in proliferation among the three groups were observed when either $CD4^+CD25^-$ T-cells were cultured alone

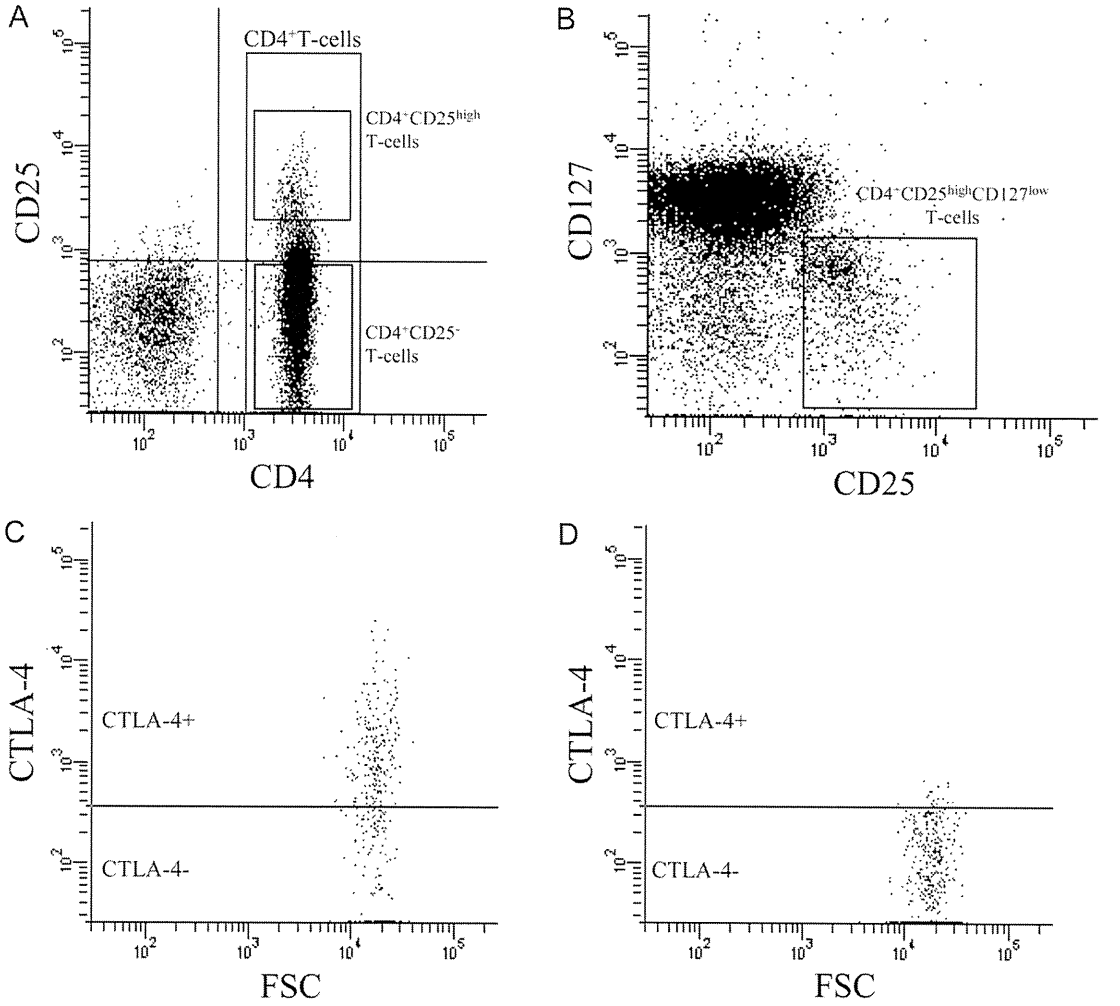

Fig. 1. Flow cytometric analysis of intracellular CTLA-4. Flow cytometric analysis of intracellular CTLA-4 from fresh peripheral blood. Representative plots showed one healthy control sample gated on lymphocytes and $CD3^+$ T-cells showing CD4 and CD25 staining (A) and CD25 and CD127 staining (B), and intracellular staining for CTLA-4 (C), as well as the isotype control (D) by gating on $CD4^+$ T-cells or $CD4^+CD25^{high}$ T-cells ($CD25^+$ top 1% of $CD3^+$ T-cells) or $CD4^+CD25^-$ T-cells.

(0:1 ratio) or $CD4^+CD25^{high}$ T-cells were cultured alone (1:0 ratio) (Fig. 4A). The suppressive function of the $CD4^+CD25^{high}$ T-cells did not differ among the group with fulminant type 1 diabetes [median 90%, range 70–98%], the group with type 1A diabetes [median 78%, range 64–95%] and the normal control subjects [median 84.5%, range 46–95%] (Fig. 4B).

3.6. Correlation between the proliferation and the levels of CTLA-4 in $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T-cells

When we investigated the correlation between proliferation and the levels of CTLA-4 in $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T-cells in 18 subjects (7 fulminant type 1 diabetes, 5 type 1A diabetes and 6 control subjects), there was a significant negative correlation in $CD4^+CD25^-$ T-cells [$r = -0.4473$, $P < 0.05$] (Fig. 5A), but not in $CD4^+CD25^{high}$ T-cells (Fig. 5B).

3.7. Correlation between CTLA-4 polymorphisms and the levels of CTLA-4 in $CD4^+$ helper T-cells

The frequency of the CTLA-4 polymorphisms (exon1 *+49* and *CT60*) is shown in Table 2. There was no correlation between those polymorphisms and the levels of CTLA-4 expression in $CD4^+$ helper T-cells either in patients with fulminant type 1 diabetes or in all subjects considered together in this study.

4. Discussion

We have clearly shown that intracellular CTLA-4 expression was reduced in $CD4^+$ helper T-cells from patients with fulminant

Table 2
CTLA-4 polymorphisms in fulminant type 1 diabetes, type 1A diabetes, type 2 diabetes and healthy control subjects.

	Fulminant type 1 diabetes	Type 1A diabetes	Type 2 diabetes	Control subjects
+49 G>A				
n	10	11	7	13
AA	0(0)	0(0)	1(14.2)	3(23.1)
AG	3(30.0)	6(54.5)	3(42.9)	3(23.1)
GG	7(70.0)	5(45.5)	3(42.9)	7(53.8)
CT60 G>A				
n	10	11	7	13
AA	0(0)	0(0)	0(0)	2(15.4)
AG	1(10.0)	2(18.2)	1(14.3)	3(61.5)
GG	9(90.0)	9(81.8)	6(85.7)	8(61.5)

Data are *n* (%).