Endocrine Journal 2000, **47** (6), 753–761

HLA-Associated Cellular Response to GAD in Type 2 Diabetes with Antibodies to GAD

MICHIAKI FUKUI*,**, NAOTO NAKAMURA*, KOJI NAKANO*, SHIZUO KAJIYAMA***, SATOSHI MATSUO#, HIROSHI OBAYASHI##, MITSUHIRO OHTA##, MASAKO SHIGETA*, HIROFUMI SHIGETA*, YOSHIHIRO KITAGAWA*,** AND MOTOHARU KONDO*

* *The First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto 602–8566, Japan*
** *Osaka General Hospital of West Japan Railway Company, Osaka 545–0002, Japan*
*** *Kyoto City Hospital, Kyoto 604–8845, Japan*
\# *Kyoto Second Red Cross Hospital, Kyoto 602–8026, Japan*
\#\# *Clinical Research Center, Utano National Hospital, Kyoto 616–8255, Japan*

Abstract. Proliferative response of peripheral blood mononuclear cells (PBMC) to glutamic acid decarboxylase (GAD), which has been reported in patients with type 1 diabetes, was measured in type 2 diabetes, especially in patients with antibodies to GAD initially diagnosed as having type 2 diabetes (anti-GAD$^+$ type 2 diabetes). We studied 12 patients with type 1 diabetes, 22 with anti-GAD$^+$ type 2 diabetes, 31 with type 2 diabetes who were negative for anti-GAD (anti-GAD$^-$ type 2 diabetes), and 30 healthy control subjects for cellular responses in vitro to GAD. The mean stimulation index (SI) in response to GAD was significantly higher in type 1 diabetes than in anti-GAD$^-$ type 2 diabetes or healthy controls (1.47 ± 0.35 vs. 1.11 ± 0.35, $P<0.05$, and 1.06 ± 0.07, $P<0.05$, respectively). The mean SI in response to GAD in anti-GAD$^+$ type 2 diabetes was significantly higher than in healthy controls (1.36 ± 0.50 vs. 1.06 ± 0.07, $P<0.05$). In anti-GAD$^+$ type 2 diabetes, the mean SI in response to GAD was significantly higher in patients with alleles susceptible to type 1 diabetes (HLA-DRB1*0405 and 0901) than those without susceptible alleles (1.55 ± 0.60 vs. 1.12 ± 0.16, $P<0.05$). All but one patient with a positive response to GAD had developed insulin deficiency ($P<0.01$ vs. nonresponders). In conclusion, we observed a significantly greater proliferative response to GAD in patients with anti-GAD$^+$ type 2 diabetes, especially those with alleles susceptible to type 1 diabetes, and those responses may be a useful predictive marker for later development of insulin deficiency in anti-GAD$^+$ type 2 diabetes.

Key words: Cellular proliferation, Type 2 diabetes, HLA, Autoantibody, GAD

(*Endocrine Journal* **47**: 753–761, 2000)

ALTHOUGH autoantibodies to islet cell antigens such as glutamic acid decarboxylase (GAD), insulin, BSA, and ICA512 have been described as useful markers identifying autoimmune diabetes that predict later development of type 1 diabetes [1–3], no definite pathogenetic role has yet been assigned to them. Since autoimmune destruction of islet cells is mediated predominantly through autoreactive T-cells [4–6], measurement of cellular immune responses against islet cell autoantigens may be more informative than serum concentrations of autoantibodies to the same antigens.

GAD is a major autoantigen in human type 1 diabetes [7, 8] as well as in the nonobese diabetic (NOD) mouse [9–12], a model of type 1 diabetes. Presently, autoantibodies to GAD (anti-GAD) are the most useful marker for the diagnosis and prediction of type 1 diabetes [13–15]. Several recent reports have identified autoimmune markers such as anti-GAD in

Received: August 10, 2000
Accepted: September 29, 2000
Correspondence to: Michiaki FUKUI, M.D., The Department of Endocrinology and Hematology, Osaka General Hospital of West Japan Railway Company, 1–3–5 Minami, Tennoji-cho, Abeno-ku, Osaka 545–0002, Japan

patients initially diagnosed as having type 2 diabetes as well as type 1 diabetes, and these type 2 diabetic patients tended to develop insulin deficiency within a short period [16–21]. In addition, we have found that a combination of an immunologic marker (anti-GAD) and a genetic marker (HLA-DRB1) could more accurately predict progression to insulin deficiency than either marker alone [22]. Proliferation of peripheral blood mononuclear cells (PBMC) in response to GAD has been reported in type 1 diabetes [23–26], but no investigations have described the PBMC response to GAD in type 2 diabetes, especially in patients with anti-GAD initially diagnosed as having type 2 diabetes (anti-GAD$^+$ type 2 diabetes). We therefore studied the PBMC response to islet cell antigens, including GAD, in anti-GAD$^+$ type 2 diabetes. We further evaluated cellular immune response to GAD in anti-GAD$^+$ type 2 diabetes according to HLA types with and without alleles susceptible to type 1 diabetes (DRB1*0405 and 0901). The aim of this study was to determine whether PBMC response to GAD was enhanced in anti-GAD$^+$ type 2 diabetes, particularly in patients with alleles susceptible to type 1 diabetes, and whether such reactivity could serve as a prognostic marker in anti-GAD$^+$ type 2 diabetes.

Materials and Methods

Subjects

We studied cellular responses to islet cell antigens such as GAD, insulin, and BSA in 12 patients with type 1 diabetes, 22 with anti-GAD$^+$ type 2 diabetes, and 31 with type 2 diabetes negative for anti-GAD (anti-GAD$^-$ type 2 diabetes), as well as in 30 healthy control subjects (16 men and 14 women; age, 51.4±10.7 years). Clinical profiles of patients with type 1 diabetes, anti-GAD$^+$ type 2 diabetes, and anti-GAD$^-$ type 2 diabetes are shown in Table 1. Additionally, respective features of anti-GAD$^+$ type 2 diabetes with (n=12) and without (n=10) the alleles susceptible to type 1 diabetes (DRB1*0405 and 0901) were as follows: BMI, 21.0±3.6 and 21.0±1.2 kg/m^2; age, 61.8±15.1 and 63.6±7.2 years; age at onset, 49.9±12.5 and 43.4±6.5 years; duration of diabetes, 11.8±9.3 and 20.2±5.3 years; and fasting plasma C-peptide, 0.36±0.31 and 0.38±0.29 nmol/l, respectively. Type 2 diabetes was defined as well controlled by diet and oral hypoglycemic agents for at least 1 year, and type 1 diabetes was defined as ketosis-prone at the time of diagnosis because of insulin deficiency with an abrupt onset. Diabetic patients studied were all unrelated Japanese, and were recruited from hospitals in Kyoto, Japan. All patients gave informed consent to study participation. Pancreatic beta cell function was evaluated by the fasting plasma C-peptide concentrations, and insulin deficiency was defined as a fasting plasma C-peptide concentration less than 0.16 nmol/l.

Measurement of anti-GAD

In 1998, we remeasured anti-GAD in type 2 diabetes found to be positive for anti-GAD from 1995 to 1997. A radioimmunoprecipitation assay with recombinant human GAD65 and a commercial anti-GAD antibody assay kit (Cosmic, Tokyo, Japan) was used according to the manufacture's recommen-

Table 1. Clinical profiles of patients with type 1, anti-GAD$^+$ type 2, and anti-GAD$^-$ type 2 diabetes

	Type 1	Anti-GAD$^+$ type 2	Anti-GAD$^-$ type 2
Subjects (n)	12	22	31
Males/females (n)	3/9	10/12	13/18
Body mass index (kg/m^2)	19.6 ± 0.4	21.0± 2.8[a]	22.0± 3.3[a]
Age (years)	21.3 ± 7.2	62.5±11.9[b]	66.9± 9.8[b]
Age at onset (years)	17.6 ±14.9	46.9±10.6[b]	46.4±10.4[b]
Duration of diabetes (years)	3.7 ± 1.6	15.2± 8.9[b]	20.5± 9.5[b,c]
Fasting plasma C-peptide (nmol/L)	0.03± 0.0	0.4± 0.3[b]	0.4± 0.3[b]

Data are mean±SD. [a] $P<0.05$ vs. type 1; [b] $P<0.01$ vs. type 1; [c] $P<0.05$ vs. anti-GAD$^+$ type 2.

dation [27]. In brief, we mixed 20 μl of undiluted test sera and 50 μl of the tracer reagent, ^{125}I-labeled GAD solution, in a 12×75 mm plastic test tube that was incubated for 2 h at room temperature. We then added 50 μl of precipitation reagent containing Protein A for the immunoprecipitation assay. After incubating this mixture for 1 h at room temperature, we added 1 ml of the assay buffer, pelleted the precipitate by centrifugation at 1500 g for 30 min at 4°C, and aspirated the supernatant liquid. Radioactivity of the precipitate was then measured in a gamma counter. The level of GAD antibodies in the sample was determined by comparison with a calibration curve. Serum samples were considered positive if they contained >1 U of GAD antibodies, which is 4 SD above the mean value for 98 heathy control subjects.

HLA typing

HLA-DRB1 allele typing was performed by the polymerase chain reaction-low ionic strength-single strand conformation polymorphism (PCR-LIS-SSCP) method of Maruya *et al.* [28]. Briefly, genomic DNA was extracted from peripheral blood leukocyte. The DNA (100 ng) was amplified by PCR with 1.25 units of Taq DNA polymerase (Perkin-Elmer Cetus). The second exon of the DRB1 gene was amplified using seven sets of group-specific primers. One microliter of PCR product was added to 20 μl of LIS solution. The mixture was then incubated and applied to a polyacrylamide gel, and electrophoresis was carried out. Single-stranded DNA in the gel was detected by silver staining.

PBMC proliferative response

Peripheral blood mononuclear cells were isolated from heparinized whole blood, taken simultaneously with serum for measurement of anti-GAD, by Ficoll-Hypaque density centrifugation. Then 1×10^5 PBMC per well were cultured in flat-bottom 96-well tissue culture trays in 100 μl of RPMI 1640 containing 10% autologous serum for 7 days (95% air/5% CO_2). The cells were incubated with the following antigens/mitogens in triplicate cultures: 10 μg/ml recombinant human GAD65 (RSR, Cardiff, UK); 10 μg/ml insulin; 10 μg/ml BSA; 10 μg/ml phytohemagglutin (PHA), and 10 μg/ml lipopolysaccaride (LPS). PHA and LPS responses were

Table 2. Frequency and levels of cellular immune responses to islet cell antigens in patients with type 1, anti-GAD$^+$ type 2, Anti-GAD$^-$ type 2, and healthy controls

	Antigen		
Subject group	GAD	Insulin	BSA
Type 1			
Frequency	6/12 (50)[a,b]	5/12 (42)[a,c]	3/12 (25)
Level	1.47±0.35[a,d]	1.32±0.38[c,e]	1.21±0.48
Anti-GAD$^+$ type 2			
Frequency	6/22 (27)[f]	3/22 (14)	3/22 (14)
Level	1.36±0.50[d]	1.15±0.26	1.16±0.28
Anti-GAD$^-$ type 2			
Frequency	4/31 (13)	3/31 (10)	4/31 (13)
Level	1.11±0.35	0.99±0.32	1.01±0.30
Control			
Frequency	0/30 (0)	0/30 (0)	0/30 (0)
Level	1.06±0.07	0.96±0.09	1.02±0.09

Data are the mean±SD of stimulation indices. Frequency values within parentheses represent the percentage of positive individuals for the indicated antigen. [a] $P<0.05$ vs. anti-GAD$^-$ type 2; [b] $P<0.0005$ vs. control; [c] $P<0.005$ vs. control; [d] $P<0.05$ vs. control; [e] $P<0.01$ vs. anti-GAD$^-$ type 2; [f] $P<0.01$ vs. control.

measured after 4 days.

The procedure for recombinant GAD65 production has been described previously [29]. N-terminally modified GAD65, lacking N-terminal amino acids (2 to 45 inclusive), was used as a stimulant because of its stability in solution, whereas affinity-purified full-length GAD65 tends to precipitate out of solution. Tetrazolium salt reduction assay (MTT assay) was performed to measure cell proliferation, which is known to reproducibly and accurately quantitate cell number and favorably compare with [^{3}H] thymidine assay [30]. Briefly, incubated cells described as above were loaded with 15 μl of MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) and incubated for a further 4 h. One hundred μl acidified beta-isopropanol was then added to the medium and the solution was left for 1 h at room temperature for color development, before being read by ELISA reader (570 nm filter).

Cellular proliferation was expressed as the stimulation index (SI), equal to mean counts incorporated in the presence of antigen divided by mean counts incorporated in the absence of antigen. A positive cellular immune response was defined as an SI exceeding the mean plus 4 SD of healthy control responses for test antigens (1.4 for GAD, insulin, and BSA stimulation).

Statistical analysis

Analysis of differences between groups was performed using analysis of variance (ANOVA) with Scheffe's F test. Chi-squared analysis, with Yates' correction when appropriate, and Fisher's exact test were used to determine the statistical significance of differences between group frequencies. Relationships between humoral and cellular immune responses were evaluated by Spearman correlation. A level of $P<0.05$ was accepted as statistically significant.

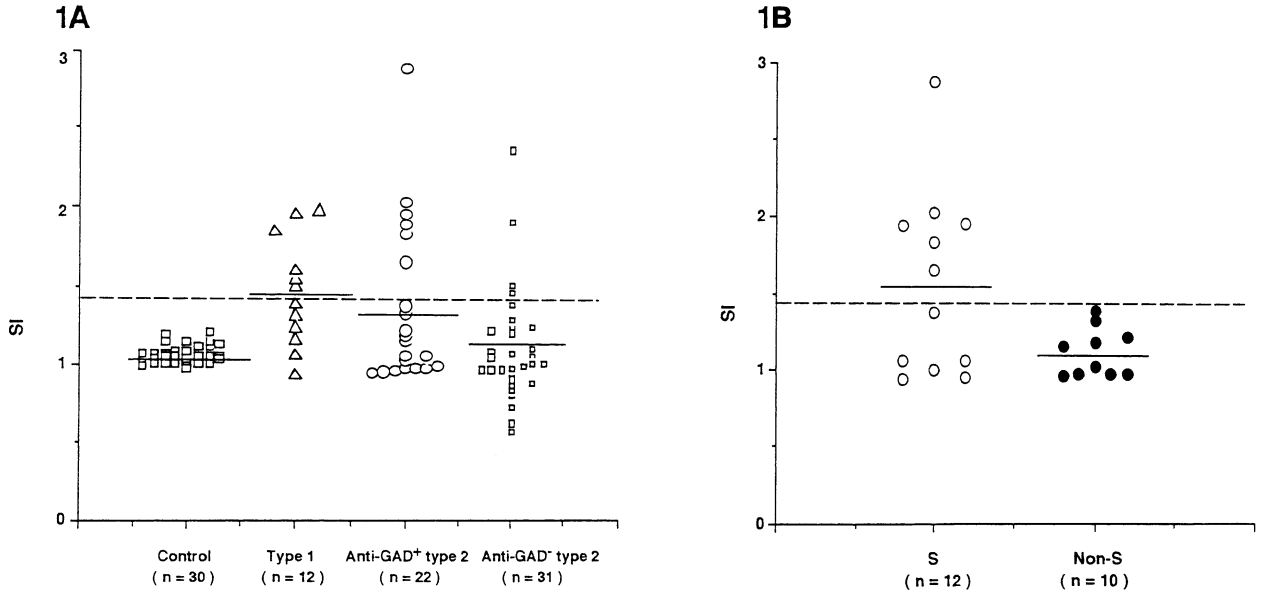

Fig. 1. **(1A)**. Proliferation of PBMC in response to GAD in healthy controls and in patients with type 1 diabetes, anti-GAD$^+$ type 2 diabetes, and anti-GAD$^-$ type 2 diabetes. Dashed lines represent thresholds for positive reactivity. Solid bars indicate group means. The mean SI to GAD in type 1 diabetes was significantly higher than in anti-GAD$^-$ type 2 diabetes or healthy controls (1.47 ± 0.35 vs. 1.11 ± 0.35; $P<0.05$, and 1.06 ± 0.07; $P<0.05$, respectively). The mean SI to GAD in anti-GAD$^+$ type 2 diabetes was significantly higher than in healthy controls (1.36 ± 0.50 vs. 1.06 ± 0.07; $P<0.05$). **(1B)**. Proliferative responses to GAD in patients with anti-GAD$^+$ type 2 diabetes with (○) alleles susceptible to type 1 diabetes (HLA-DRB1*0405 and 0901) (S) and without (●) alleles susceptible to type 1 diabetes (Non-S). The mean SI in response to GAD was significantly higher in anti-GAD$^+$ type 2 diabetes with alleles susceptible to type 1 diabetes than in patients without those alleles (1.55 ± 0.60 vs. 1.12 ± 0.16; $P<0.05$).

Results

PBMC proliferative response to islet cell antigens

Proliferative responses of PBMC to various antigens are shown in Table 2. The mean SI to GAD in type 1 diabetes was significantly higher than in anti-GAD$^-$ type 2 diabetes or healthy controls. Furthermore, the mean SI to GAD in anti-GAD$^+$ type 2 diabetes was significantly higher than in healthy controls. No differences in proliferative response to GAD were observed between type 1 diabetes and anti-GAD$^+$ type 2 diabetes. The proliferative response to insulin was higher in type 1 diabetes than in anti-GAD$^-$ type 2 diabetes or healthy controls. In contrast, no differences in proliferative response to BSA were observed between groups. We found an enhanced proliferative response to GAD (SI$>$1.4) in 6 of 12 type 1 diabetes ($P<0.0005$ vs. controls) and in 6 of 22 anti-GAD$^+$ type 2 diabetes ($P<0.01$ vs. controls). An enhanced cellular response to insulin was observed in 5 of 12 type 1 diabetes ($P<0.005$ vs. controls). No PBMC of patients with anti-GAD$^+$ type 2 diabetes responded to more than two antigens.

The distribution of the SI in response to GAD in patients with type 1 diabetes, anti-GAD$^+$ type 2 diabetes, and anti-GAD$^-$ type 2 diabetes together with those in healthy controls is shown in Fig. 1A. The distribution of the SI in response to GAD among patients with anti-GAD$^+$ type 2 diabetes is compared between patients with and without alleles susceptible to type 1 diabetes (DRB1*0405 and 0901) in Fig. 1B. The mean SI in response to GAD was sig-

Table 3. Clinical profiles of patients with anti-GAD$^+$ type 2 diabetes

	Patient number	Age (years)	Age at onset (years)	Disease duration (years)	BMI (kg/m^2)	sCPR (nmol/l)	Current treatment	GAD Ab (U)	GAD Ab re (U)	HLA-DRB1 genotype	SI
Responder	1	52	37	15	22	0.03	Insulin	1.1	21	0405/0802	2.88
	2	79	52	27	18	0.1	Insulin	1.6	0	0803/0901	2.02
	3	38	34	4	21	0.03	Insulin	56	5.4	0901/0901	1.95
	4	70	67	3	15	0.1	Insulin	179.6	53.3	0405/0901	1.94
	5	60	50	10	23	0.7	Insulin	2.9	3.4	0901/1501	1.83
	6	68	64	4	25	0.03	Insulin	1514	4660	0405/0901	1.65
Nonresponder	7	51	28	23	21	1.1	SU	2.5	0	0101/1502	1.39
	8	62	51	11	27	1	SU	2	0	0406/0901	1.37
	9	78	52	26	22	0.13	Insulin	1.8	0.7	1302/1502	1.32
	10	63	41	22	21	0.37	SU	3.5	0	1302/1405	1.23
	11	66	40	26	19	0.33	Insulin	2.3	0	0803/1501	1.22
	12	58	49	9	22	0.37	Insulin	1.9	0	0802/1302	1.18
	13	66	45	21	21.5	0.5	SU	2	0	0403/0408	1.18
	14	65	44	21	21	0.03	Insulin	1.3	0	0406/1403	1.16
	15	60	43	17	20.5	0.23	SU	5	0	1101/1401	1.13
	16	59	41	18	16	0.6	SU	1.4	0	0901/0901	1.06
	17	64	59	5	19	0.33	SU	1.1	7.6	0405/1302	1.06
	18	60	47	13	19	0.53	SU	13.3	0	0802/1202	1.03
	19	33	30	3	26	0.3	Insulin	16	9.1	0405/1302	1
	20	69	45	24	23	0.17	Insulin	1.3	0	0410/1302	0.97
	21	84	67	17	20	0.43	SU	3.3	20.3	0901/0901	0.95
	22	72	57	15	19.5	0.63	SU	6	4	0901/1101	0.94

Responder, patient with a positive cellular response to GAD; sCPR, fasting plasma C-peptide concentration; SU, sulfonylurea; GAD Ab, original level of antibodies to GAD; GAD Ab re, reexamined level of antibodies to GAD (1998); SI, stimulation index in response to GAD.

nificantly higher in anti-GAD$^+$ type 2 diabetes with alleles susceptible to type 1 diabetes than in patients without those alleles (1.55 ± 0.60 vs. 1.12 ± 0.16; $P<0.05$). Proliferative response to PHA and LPS (both positive controls) showed no differences between groups.

Profile of anti-GAD$^+$ type 2 diabetes

Clinical profiles of patients with anti-GAD$^+$ type 2 diabetes are shown in Table 3. All six patients whose PBMC responded to GAD had alleles susceptible to type 1 diabetes (DRB1*0405 and 0901). In addition, all but one patient with a positive cellular response to GAD had developed insulin deficiency ($P<0.01$ vs. cases with a negative response to GAD). All patients with anti-GAD$^+$ type 2 diabetes without alleles susceptible to type 1 diabetes were negative for anti-GAD in 1998. No significant relationship was seen between humoral and cellular immune response to GAD ($P=0.285$, Fig. 2).

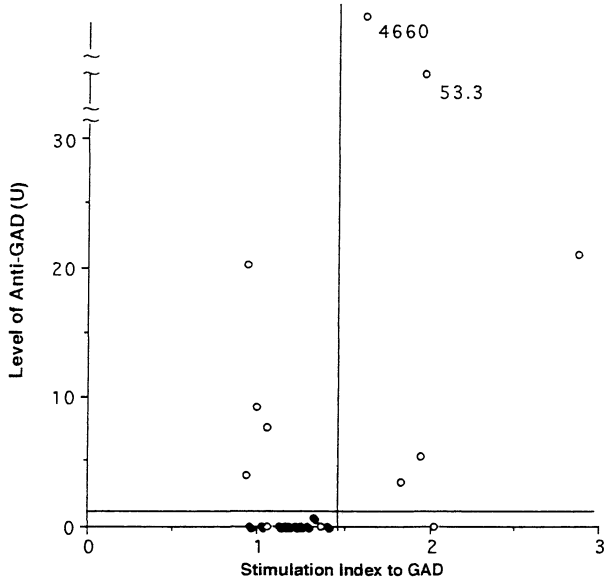

Fig. 2. Relationship between levels of anti-GAD and stimulation indices in response to GAD antigen in patients with anti-GAD$^+$ type 2 diabetes with (○) and without (●) alleles susceptible to type 1 diabetes. The lines represent thresholds for positive reactivity. No significant relationship was seen between humoral and cellular immune response to GAD ($P=0.285$).

Discussion

Several previous reports have described cellular immune response to islet cell antigens such as GAD, insulin, BSA, and others in patients with type 1 diabetes. To our knowledge, no reports have addressed cellular immune response to islet cell antigens in type 2 diabetes, especially in patients with anti-GAD. Autoimmune destruction of islet cells is mediated predominantly through autoreactive T cells, and measurement of cellular immune response against islet cell autoantigens may be more informative than determining the level of autoantibodies to these antigens, even though anti-GAD have been identified as a useful predictive marker for subsequent development of insulin deficiency in patients with type 2 diabetes. Accordingly, we first evaluated cellular immune response to GAD in anti-GAD$^+$ type 2 diabetes, finding it to be increased in anti-GAD$^+$ type 2 diabetes as well as type 1 diabetes, especially in patients with alleles susceptible to type 1 diabetes (DRB1 *0405 and 0901).

Brooks-Worrell *et al.* investigated whether type 2 diabetes positive for islet autoantibodies have PBMC responses to islet proteins [31]. They reported that anti-GAD$^+$ type 2 diabetes also demonstrated PBMC responses to islet proteins, but overall magnitude of the responses to blot sections was significantly less in anti-GAD$^+$ type 2 diabetes compared with that of type 1 diabetes. In our study, PBMC of patients with anti-GAD$^+$ type 2 diabetes responded to GAD, one of the major antigens in islet proteins, but not to insulin and BSA. The magnitude of the responses to GAD was similar in anti-GAD$^+$ type 2 diabetes compared with type 1 diabetes, which might be due to long duration after onset of type 1 diabetes (3.7 ± 1.6 years) compared with the subjects (within 1 year after onset) in their report.

Interestingly, all six patients whose PBMC responded to GAD had alleles susceptible to type 1 diabetes. In addition, all but one of them with positive response to GAD had developed insulin deficiency. One non-insulin deficient anti-GAD$^+$ type 2 diabetes with enhanced response to GAD had a unique history. He had been treated with low-dose insulin in the early stage of illness, which may have protected him from development of insulin deficiency as previously reported [32]. Another speculation might be that this patient had a dominantly acting

protective allele (DRB1*1501) in addition to the susceptible allele.

We have previously reported that a combination of an immunologic marker (anti-GAD) and a genetic marker (HLA-DRB1) has exceptional prognostic value for anti-GAD$^+$ type 2 diabetes. Moreover, cellular immune response to GAD may be predictive for development of insulin deficiency in anti-GAD$^+$ type 2 diabetes. This marker may be useful in selecting patients for immunotherapy aimed at preventing autoimmune destruction of insulin producing islet beta cells [33, 34].

Anti-GAD$^+$ type 2 diabetes responded only to GAD, while type 1 diabetes responded to insulin as well as GAD. This finding is compatible with previous data, although there exists the difference between cellular and humoral autoimmunity and cellular immunity was reported to be predominant [35], suggesting that patients who are positive for two or more antibodies (such as anti-GAD, insulin autoantibodies, and IA-2 autoantibodies) are at higher risk for development of type 1 diabetes [17, 36–38].

We used a nonradioactive MTT assay to determine cellular response to islet cell antigens. SI values above the cutoff were somewhat lower than the conventional proliferation assay using radioactive [^{3}H] thymidine, while the MTT assay is advantageous because it produces stable data and is nonradioactive, giving results that compare favorably with those of [^{3}H] thymidine assays [30].

In the present study, a stable recombinant human GAD65 of nearly full-length was used to stimulate PBMC. We plan to evaluate PBMC proliferative response to GAD peptide according to HLA in the future.

Surprisingly, all anti-GAD$^+$ type 2 diabetes without alleles susceptible to type 1 diabetes had become negative for anti-GAD instead of maintaining persistent levels in patients with alleles susceptible to type 1 diabetes. The low-grade nature of the immune response to GAD may lead to beta cell preservation in anti-GAD$^+$ type 2 diabetes without susceptible alleles.

Instead of an inverse correlation of levels of anti--GAD and proliferative responses to GAD antigen as observed in first-degree relatives of type 1 diabetes by Harrison *et al.* [35], we found no correlation between levels of anti-GAD and SI to GAD antigen in anti-GAD$^+$ type 2 diabetes.

Enhanced proliferative responses to GAD were observed even in some anti-GAD$^-$ type 2 diabetes, which suggests either that the cellular immune response is a more sensitive marker than autoantibodies test or that these previously positive patients turned negative for anti-GAD instead of experiencing ongoing autoimmune islet cell destruction. Proliferative responses to islet cell antigens have been reported even in control subjects. We found no proliferative responses in controls, possibly because of population differences. Autoimmune diabetes is less prevalent in Japan than in Western countries [39].

Vaarala *et al.* have reported that the median SI to beta-lactoglobulin did not differ significantly according to HLA-DQB1 risk alleles in type 1 diabetes [40]. In contrast, our data clearly demonstrated an enhanced cellular immune response to GAD in anti-GAD$^+$ type 2 diabetes with certain HLA-DRB1 alleles. The reason for the difference is unclear, but it may involve differences in clinical features of diabetes, HLA type, or islet cell antigens used for stimulation.

Unfortunately our study was cross-sectional in nature, and prospective longitudinal studies will be needed to clarify our observations. However, to our knowledge, they represent the first characterization of cellular immune response to islet cell antigens, especially GAD, in anti-GAD$^+$ type 2 diabetes. Recently a new classification of diabetes mellitus has been proposed by the American Diabetes Association [41], and it will be interesting to evaluate HLA type and cellular immune responses to islet cell antigens in subclassification of autoimmune diabetes mellitus.

In conclusion, we observed a significantly greater proliferative response to GAD in patients with anti-GAD$^+$ type 2 diabetes, especially those with alleles susceptible to type 1 diabetes. Cell responses appear to be a promising predictive marker for later development of insulin deficiency in anti-GAD$^+$ type 2 diabetes.

References

1. Myers MA, Rabin DU, Rowley MJ (1995) Pancreatic islet cell cytoplasmic antibody in diabetes is represented by antibodies to islet cell antigen 512 and glutamic acid decarboxylase. *Diabetes* 44: 1290–1295.
2. Landin-Olsson M, Palmer JP, Lernmark A, Blom L, Sundkvist G, Nystrom L, Dahlquist G (1992) Predictive value of islet cell and insulin autoantibodies for Type 1 (insulin-dependent) diabetes mellitus in a population-based study of newly-diagnosed diabetic and matched control children. *Diabetologia* 35: 1068–1073.
3. Savola K, Bonifacio E, Sabbah E, Kulmala P, Vahasalo P, Karjalainen J, Tuomilehto WE, Merilainen J, Akerblom HK, Knip M (1998) IA-2 antibodies—a sensitive marker of IDDM with clinical onset in childhood and adolescence. Childhood diabetes in Finland study group. *Diabetologia* 41: 424–429.
4. Haskins K, Wegmann D (1996) Diabetogenic T-cell clones. *Diabetes* 45: 1299–1305.
5. Roep BO (1996) T-cell responses to autoantigens in IDDM. *Diabetes* 45: 1147–1156.
6. Honeyman MC, Stone N, Aizpurua H, Rowley MJ, Harrison LC (1997) High T cell responses to the glutamic acid decarboxylase (GAD) isoform 67 reflect a hyperimmune state that precedes the onset of insulin-dependent diabetes. *J Autoimmun* 10: 165–173.
7. Atkinson MA, Maclaren NK (1993) Islet cell autoantigens in insulin-dependent diabetes. *J Clin Invest* 92: 1608–1616.
8. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK (1994) Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. *J Clin Invest* 94: 2125–2129.
9. Zekzer D, Wong FS, Ayalon O, Millet I, Altieri M, Shintani S, Solimena M, Sherwin RS (1998) GAD-reactive CD4+ Th1 cells induce diabetes in NOD /SCID mice. *J Clin Invest* 101: 68–73.
10. Tisch R, Yang XD, Liblau RS, McDevitt HO (1994) Administering glutamic acid decarboxylase to NOD mice prevents diabetes. *J Autoimmun* 7: 845–850.
11. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO (1993) Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature* 366: 72–75.
12. Tian J, Clare SM, Herschenfeld A, Middleton B, Newman D, Mueller R, Arita S, Evans C, Atkinson MA, Mullen Y, Sarvetnick N, Tobin AJ, Lehmann PV (1996) Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* 2: 1348–1353.
13. Hagopian WA, Karlsen AE, Gottsater A, Landin OM, Grubin CE, Sundkvist G, Petersen JS, Boel E, Dryberg T, Lernmark A (1993) Quantitative assay using recombinant human islet glutamic acid decarboxylase (GAD65) shows that 64K autoantibody positivity at onset predicts diabetes type. *J Clin Invest* 91: 368–374.
14. Tuomilehto J, Zimmet PZ, Mackay IR, Koskela P, Vidgren G, Toivanen L, Tuomilehto WE, Kohtamaki K, Stengard J, Rowley MJ (1994) Antibodies to glutamic acid decarboxylase as predictors of insulin-dependent diabetes mellitus before clinical onset of disease. *Lancet* 343: 1383–1385.
15. Atkinton MA, Maclaren NK, Scharp DW, Lacy PE, Riley WJ (1990) 64000 Mr autoantibodies as predictors of insulin-dependent diabetes. *Lancet* 335: 1357–1360.
16. Kobayashi T, Nakanishi K, Okubo M, Murase T, Kosaka K (1996) GAD antibodies seldom disappear in slowly progressive IDDM. *Diabetes Care* 19: 1031.
17. Abiru N, Takino H, Yano M, Kawasaki E, Yamasaki H, Yamaguchi Y, Akazawa S, Nagataki S (1996) Clinical evaluation of non-insulin-dependent diabetes mellitus patients with autoantibodies to glutamic acid decarboxylase. *J Autoimmun* 9: 683–688.
18. Rowley MJ, Mackay IR, Chen Q, Knowles WJ, Zimmet PZ (1992) Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus. *Diabetes* 41: 548–551.
19. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, Lang DA (1993) Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabetic Med* 11: 299–303.
20. Tuomi T, Groop LC, Zimmet PZ, Rowley MJ, Knowles W, Mackay IR (1993) Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin-dependent onset of disease. *Diabetes* 42: 359–362.
21. Niskanen LK, Tuomi T, Karjalainen J, Groop LC, Uusitupa MI (1995) GAD antibodies in NIDDM: ten-year follow-up from the diagnosis. *Diabetes Care* 18: 1557–1565.
22. Fukui M, Nakano K, Maruya E, Saji H, Ohta K, Ohta M, Obayashi H, Mori H, Kajiyama S, Wada S, Shigeta H, Kitagawa Y, Nakamura N, Kondo M (1997) Diagnostic significance of antibodies to glutamic acid decarboxylase in Japanese diabetic patients with secondary oral hypoglycemic agents failure. *Clin Immunol Immunopathol* 85: 182–186.
23. Brooks-Worrell BM, Starkebaum GA, Greenbaum CJ, Palmer JP (1996) Peripheral blood mononuclear

cells of insulin-dependent diabetic patients respond to multiple islet cell proteins. *J Immunol* 157: 5668–5674.

24. Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF, Erlander MG, Tobin AJ, Maclaren NK (1992) Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet* 339: 458–459.
25. Schloot NC, Roep BO, Wegmann DR, Yu L, Wang TB, Eisenbarth GS (1997) T-cell reactivity to GAD65 peptide sequences shared with coxsackie virus protein in recent-onset IDDM, post-onset IDDM patients and control subjects. *Diabetologia* 40: 332–338.
26. Hummel M, Bello ID, Ziegler AG (1996) Relation between cellular and humoral immunity to islet cell antigens in Type 1 diabetes. *J Autoimmun* 9: 427–430.
27. Ohta M, Obayashi H, Takahashi K, Kitagawa Y, Nakano K, Matsuo S, Nishimura M, Itoh N, Ohta K (1996) Radioimmunoprecipitation assay for glutamic acid decarboxylase antibodies evaluated clinically with sera from patients with insulin-dependent diabetes mellitus. *Clin Chem* 42: 1975–1978.
28. Maruya E, Saji H, Yokoyama S (1996) PCR-LIS-SSCP (low ionic strength single-stranded conformation polymorphism)—a simple method for high-resolition allele typing of HLA-DRB1, -DQB1, and -DPB1. *Genome Res* 6: 51–57.
29. Powell M, Prentice L, Awata T, Kato R, Sawicka J, Tanaka H, Petersen V, Munkley A, Morgan S, Smith BR, Furmaniak J (1996) Glutamic acid decarboxylase autoantibody assay using ^{125}I-labelled recombinant GAD65 produced in yeast. *Clin Chim Acta* 256: 175–188.
30. Hussain RF, Nouri AME, Oliver RTD (1993) A new approach for measurement of cytotoxicity using colorimetric assay. *J Immunol Methods* 160: 89–96.
31. Brooks-Worrell BM, Juneja R, Minokadeh A, Greenbaum CJ, Palmer JP (1999) Cellular immune responses to human islet proteins in antibody-positive type 2 diabetic patients. *Diabetes* 48: 983–988.
32. Kobayashi T, Nakanishi K, Murase T, Kosaka K (1996) Small doses of subcutaneous insulin as a strategy for preventing slowly progressive beta-cell failure in islet cell antibody-positive patients with clinical features of NIDDM. *Diabetes* 45: 622–626.
33. Elliott RB, Chase HP (1991) Prevention or delay of Type 1 (insulin-dependent) diabetes mellitus in children using nicotinamide. *Diabetologia* 34: 362–365.
34. Keller RJ, Eisenbarth GS, Jackson RA (1993) Insulin prophylaxis in individuals at high risk of type 1 diabetes. *Lancet* 341: 927–928.
35. Harrison LC, Honeyman MC, Deaizpurua HJ, Schmidli RS, Colman PG, Tait BD, Cram DS (1993) Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes. *Lancet* 341: 1365–1369.
36. Dittler J, Seidel D, Schenker M, Ziegler AG (1998) GADIA2-combi determination as first-line screening for improved prediction of Type 1 diabetes in relatives. *Diabetes* 47: 592–597.
37. Pietropaolo M, Peakman M, Pietropaolo SL, Zanone MM, Foley Jr. TP, Becker DJ, Trucco M (1998) Combined analysis of GAD65 and ICA512 (IA-2) autoantibodies in organ and non-organ-specific autoimmune disease confers high specificity for insulin-dependent diabetes mellitus. *J Autoimmun* 11: 1–10.
38. Seissler J, Morgenthaler NG, Achenbach P, Lampeter EF, Glawe D, Payton M, Christie M, Scherbaum WA (1996) Combined screening for autoantibodies to IA-2 and antibodies to glutamic acid decarboxylase in first degree relatives of patients with IDDM. The DENIS study group. Deutsche nikotinamid interventions-studie. *Diabetologia* 39: 1351–1356.
39. Karvonen M, Tuomilehto J, Libman I, LaPorte R, for the World Health Organization DIAMOND Project Group (1993) A review of the recent epidemiological data on the worldwide incidence of type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 36: 883–892.
40. Vaarala O, Klemetti P, Savilahti E, Reijonen H, Ilonen J, Akerblom HK (1996) Cellular immune response to cow's milk beta-lactoglobulin in patients with newly diagnosed IDDM. *Diabetes* 45: 178–182.
41. Peters AL, Schriger DL (1998) The new diagnostic criteria for diabetes: the impact on management of diabetes and macrovascular risk factors. *Am J Med* 105: 15–19S.