Immunogenetics 28: 240-246, 1988

Immuno-genetics

HLA-DQ system and insulin-dependent diabetes mellitus in Japanese: does it contribute to the development of IDDM as it does in Caucasians?

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Abstract. Fifty-six unrelated Japanese patients with insulin-dependent diabetes mellitus (IDDM) were HLA-typed, and restriction fragment length polymorphism (RFLP) analysis was performed after enzyme digestion with *Bam* HI and *Taq* I by using both DR and DQ probes. As previously reported, increased frequencies of Bw54, Cw1, DR4, and DRw53, which are in strong linkage disequilibrium in the Japanese population and make the characteristic Japanese haplotype, were confirmed. *DQw4*, a new allele of the *DQ* system recognized by the monoclonal antibody HU-46 and in linkage disequilibrium with this haplotype, presented the highest IDDM association. The RFLP analysis also showed the strongest correlation to IDDM when the DQ probe was applied. These results indicate that HLA-DQ might play the most important role in the development of IDDM in Japanese as well as in Caucasians. The correlation of DQ_β amino acid sequences strongly associated with IDDM in Japanese are discussed in this study, and contrasting results were found when such sequences were compared with those of Caucasians.

Introduction

Insulin-dependent diabetes mellitus (IDDM) is one of the most common chronic metabolic disorders in childhood (Rossini et al. 1985). One of its main characteristics is the presence of insulitis of autoimmune origin (Bottazzo 1984). Although the number of investigations carried out to understand the genetic factors related to IDDM has increased, complete understanding of the disease requires further progress (Rimoin and Rotter 1985).

Since HLA studies began, the first associations with B8 and B15 were observed in Caucasians (Nerup et al. 1974, 1977). A stronger HLA-D-(Dw3 and/or Dw4) than B-(B8 and/or Bw15) association has also been found (Thomsen et al. 1975). Following studies within the DR antigen (Bodmer et al. 1978), the association of DR3 and DR4 was found to be more important, and the increased frequencies of B8 and B15 were thought to be a secondary effect due to the linkage disequilibrium with DR3 and DR4, respectively (Moerloose et al. 1978). The complementary effect of the susceptibility gene associated with B8-DR3 and B15-DR4 is also known, since the highest relative risk was found in *B8-DR3/B15-DR4* heterozygotes (Svejgaard et al. 1980, Svejgaard and Ryder 1981, Rotter et al. 1983).

After the discovery of the HLA-DQ antigen (Korman et al. 1985), RFLP analysis was used in IDDM studies; HLA-DQ antigens DQw2 and DQw3 proved to be most important in the development of IDDM (Cohen-Haguenauer et al. 1985, Tosi et al. 1986, Nepom et al. 1986, Festenstein et al. 1986). Hybrid DQ molecules, which only DQ-antigen heterozygotes such as *B8-DR3-DQw2/B15-DR6-DQw3* can produce, might contribute directly to the development of IDDM, since both DQ_β and DQ_α chains are polymorphic; there exists a direct transcomplementation between them (Nepom et al. 1987).

More recently, using the analysis of diabetic DNA sequences, interesting results were reported. It was observed that the amino acid at position 57 of the DQ_β chain determined IDDM susceptibility (Todd et al. 1987). Ninety percent of the IDDM patients were homozygous for nonaspartic acid (non-Asp-57), and none were homozygous for aspartic acid (Asp-57).

In Japanese as well as in Caucasian patients with IDDM there are increased frequencies of Bw54-DR4 with

Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; RFLP, restriction fragment length polymorphism; Asp, aspartic acid; Asp-57, aspartic acid at the 57th residue of the DQ_β chain; non-Asp-57, nonaspartic acid at the 57th residue of the DQ_β chain; R.R., relative risk of Woolf and Haldane

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a higher association of DRw53. This is in linkage disequilibrium with DR4 (Wakisaka et al. 1976, Okimoto et al. 1978, Moriuchi et al. 1980). Although HLA-ABC and DR associations with IDDM are well known in Japanese patients, the HLA-DQ antigen, which might have the highest association, has not been studied yet due to the absence of the DQ antigen, which is in linkage disequilibrium with Bw54-DR4 in Japanese. Recently we produced the monoclonal antibody HU-46, which can recognize the novel DQ antigen; it was designated as DQWa (Ishikawa et al. 1987) and is now officially recognized as DQw4 (W.H.O. Nomenclature Committee 1988).

This study will concentrate on whether the association of DQ antigen in relation to IDDM in Japanese, as well as in Caucasians, is more important than DR or not. Thus we first investigated the HLA phenotype of IDDM patients by the microlymphocytotoxicity test, including use of antibodies against DQw4. Then an attempt was made to analyze the genetic markers by means of RFLP analysis. In our results, although the DQ antigen appeared as the most important antigen for developing IDDM in both Japanese and Caucasians, the correlation of the amino acid sequence at the 57th residue of the DQ_β chain within IDDM is somewhat different between Japanese and Caucasians. The difference between both ethnic groups will be discussed.

Materials and methods

Patients. A total of 56 unrelated Japanese patients with IDDM, diagnosed at the Department of Pediatrics of Hokkaido University Hospital, were investigated. A random sample of 472 unrelated, healthy Japanese who were HLA typed at the Third Asia Oceania Histocompatibility Workshop were used as controls for comparison of the HLA antigen frequencies. Twenty-four HLA-phenotype-matched, unrelated Japanese (DR4, 62.5%; DR9, 33.3%; and DRw53, 79.2%) were also selected to serve as a control group for RFLP studies.

HLA typing. HLA typing was performed with the NIH standard microlymphocytotoxicity test. Trays used in this experiment were kindly provided by P. I. Terasaki, UCLA, Los Angeles, California (Lot AE6500 for HLA-ABC and lot BT6500 for HLA-DR). An additional antibody for defining DQw4, HU-46, was also used. DR4 was divided into three subspecificities according to their linkage disequilibrium with DQ antigens, i. e., DR4.1 (DRB1*0405) with DQw4, DR4.2 with DQw8, and DR4.3 (DRB1*0401) with DQw7. These correspond to Japanese subspecificities Dw15, DKT2, and Dw4, respectively (Kasahara et al. 1983).

RFLP analysis. Genomic DNA was extracted from peripheral blood-nucleated cells, obtained after HLA typing as previously reported (Takenouchi et al. 1986). RFLP analysis was performed by the standard method used at the Tenth International Histocompatibility Workshop (New York, 1987). Briefly, after endonuclease digestion for 15 h at 30 °C (*Bam* HI) or 65 °C (*Taq* I), 10 μg of digested DNA was electrophoresed on a 0.9% agarose gel at 25 V for 32 h. After electrophoresis, gels were alkaline-denatured and transferred (Southern 1975) to charge-modified nylon membranes (Gelman Sciences Inc., Ann Arbor, Michigan). After being transferred, the filters were prehybridized overnight and hybridized at 42 °C for 40 h with DQ_α, DQ_β, and DR_β probes (provided by the Tenth International Histocompatibility Workshop) labeled with [α-^{32}P] dCTP by random-primer method. The filters were then washed four times after hybridization had finished – twice with 2× saline-sodium phosphate-EDTA (SSPE) [1× SSPE: 0.15 *M* NaCl, 10 m*M* NaH_2PO_4, 1 m*M* ethylenediaminetetraacetate (EDTA)] for 5 min at room temperature, once with a mixture of 2× SSPE and 0.5% sodium dodecyl sulfate for 15 min at 65 °C, and once with 0.5× SSPE for 15 min at 65 °C – and exposed with intensifying paper to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, New York) for 20 h.

Results

HLA antigen frequencies in IDDM patients. A group of 56 unrelated Japanese patients with IDDM was HLA typed. Table 1 shows the antigen frequencies of patients compared with 472 healthy, unrelated Japanese.

Among HLA-A, HLA-B, and HLA-C antigens, three antigens showed significant differences from those of the control: Bw54 presented 46.4%, while the control had only 14.0% ($P<0.0001$, R.R. = 5.32); Cw1 had 55.4% in comparison with the control's 27.6% ($P<0.0001$, R.R. = 3.25); and Bw52 had a markedly decreased frequency of 1.8% compared with the 23.5% of the control ($P<0.0003$, R.R. = 0.06). Although the higher frequencies of A24 (76.8%), Aw33 (19.6%), B7 (21.4%), B39 (10.7%), and B44 (19.6%) were observed with relative risk that exceeded 1.5, they were not significantly different from those of the control when a corrected *P* value was applied.

Among HLA-DR antigens, DR2 showed a lower frequency compared with the control (7.1% vs 34.3%, $P<0.0001$, R.R. = 0.15), whereas DR4 had 64.3% and the control 38.5% ($P<0.005$, R.R. = 2.88). Particularly DR4.1, which is in linkage disequilibrium with Bw54, presented 58.9% versus the 24.4% of the control ($P<0.0001$, R.R. = 4.45). DR1, DRw6, and DR9 were also increased, and only DR9 showed a significant difference (48.2% vs 26.1%, $P<0.0005$, R.R. = 2.64). However, DRw53 showed the highest frequency among HLA-DR antigens (83.9% vs 64.9%, $P<0.005$, R.R. = 2.82).

In relation to the HLA-DQ antigen, the highest association of DQw4 was observed not only within the *DQ* region but also among all the antigens (75.0% vs 31.8%, $P<0.0001$, R.R. = 6.43). DQw3 was also increased in patients with the frequency of 76.8% versus 54.7% ($P<0.001$, R.R. = 2.74).

Restriction fragment length polymorphism analysis. Association of HLA to IDDM was further investigated by means of RFLP. DQ_α and DQ_β probes, after digestion with *Bam* HI and *Taq* I, were used for analysis of DNA from 47 and 56 patients, respectively. One of the representative experiments is shown in Figure 1. RFLP

Table 1. HLA antigen frequencies in Japanese patients with IDDM

HLA antigen*	Patients (%) (n=56)	Control (%) (n=472)	R.R.	P value
Aw33*	19.6	9.5	2.33	0.02†
Bw52	1.8	23.5	0.06	<0.0003
Bw54	46.4	14.0	5.32	<0.0001
Cw1	55.4	27.6	3.25	<0.0001
DR1	21.4	12.3	1.94	
DR2	7.1	34.3	0.15	<0.0001
DR3	0.0	0.0		
DR4	64.3	38.5	2.88	<0.005
DR4.1(DQw4)	58.9	24.4	4.45	<0.0001
DR4.2(DQw8)	7.1	11.9	0.57	
DR4.3(DQw7)	0.0	2.2	0.00	
DR5	14.3	18.7	0.72	
DRw6	21.4	16.1	1.42	
DR7	0.0	0.0		
DRw8	17.9	24.8	0.66	
DR9	48.2	26.1	2.64	<0.0005
DRw52	51.8	52.3	0.98	
DRw53	83.9	64.9	2.82	<0.005
DQw1	48.2	64.4	0.51	<0.02
DQw2	0.0	0.7	0.00	
DQw3	76.8	54.7	2.74	<0.001
DQw4	75.0	31.8	6.43	<0.0001

* In HLA-ABC, only significantly deviated antigens were listed
† corrected P value >0.8

patterns were in accordance with the serological results, and the fragments which appeared exclusively in Japanese IDDM patients were not observed in this study. Since the control was selected to match the HLA-DR frequencies with patients (DR4: 62.5%, DR9: 33.3%, and DRw53: 79.2% in selected control), fewer differences in the frequency of fragments between patients and the control were observed when compared with those from serological results, where only a few fragments showed significant differences. However, when it was compared with the random control, striking differences were observed in the correlation between specificities and fragments. Table 2 summarizes the RFLP results.

Hybridization with a DQ_β probe of genomic DNA digested with *Bam* HI showed one constant (10.0 kb) and four polymorphic bands (Fig. 1), where both 6.0 and 3.4 kb bands were in relation to DQw5, both with a 21.3% frequency in patients and a 12.5% frequency in the selected control (R.R. = 1.89). DQw6 was represented by a 9.2 kb band with a significantly decreased frequency of 4.3% in patients, compared with 29.2% in the control ($P<0.01$, R.R. = 0.11). DQw4 and DQw9 showed the same 11.5 kb band with the highest percentage in this group, 89.4% among patients and 75.0% in the control (R.R. = 2.81).

Using the same enzyme (*Bam* HI) and DQ_α probe, similar results were obtained. DQw1(DR1/DR2) (DQw1 associated with DR1 and/or DR2) had a 11.0 kb band and showed a slightly lower frequency among patients than in the control (27.7% vs 37.5%, R.R. = 0.51), indicating the decreased frequencies of DR2. DQw4 and DQw9, both associated to a 8.2 kb band, had the highest frequency and relative risk in this patient group compared to the control (89.4% and 70.8%, respectively, R.R. = 3.46). DQw1(DRw6), represented by a 24.0 kb band, showed a higher frequency in patients than in the control (23.4% vs 12.5%, R.R. = 2.14).

In relation to the DQ_α probe, *Taq* I confirmed the decreased frequency of DQw1(DR2), marked with a 6.7 kb band, among patients when compared to the control (3.6% vs 33.3%, $P<0.001$, R.R. = 0.07), whereas in relation to a 2.7 kb band, DQw1(DR1) was higher among patients than in the control (54.0% and 16.7%, respectively, R.R. = 1.67). DRw52, associated to a 6.4 kb band, had almost the same frequency for both groups (33.9% vs 33.3%), while a slightly increased frequency appeared in DRw53, represented by a 6.0 kb band (89.3% in patients and 82.6% in the control, R.R. = 1.75). By using a DQ_β probe after digestion with *Taq* I, the decreased DQw6 antigen frequency among patients was again evident when compared to the control (4.8% vs 21.0%, $P<0.05$, R.R. = 0.14). This was associated to a 2.8 kb band, while DQw5, represented by a 5.4 kb band, had a slightly higher

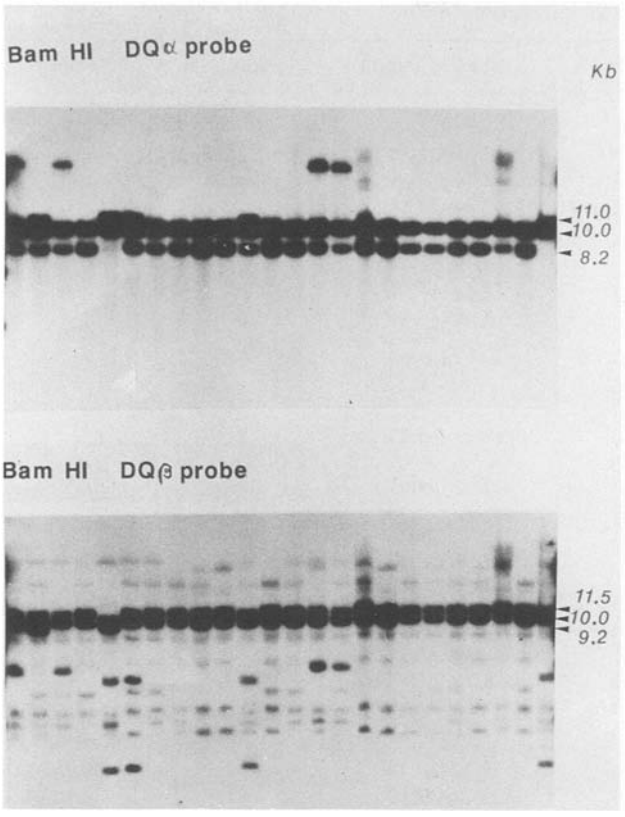

Fig. 1. RFLP patterns of 24 Japanese IDDM patients. DNA from 24 patients with IDDM were hybridized with DQ_α (*upper*) and DQ_β (*lower*) after digestion by *Bam* HI. Numbers on the *right* indicate fragment sizes

frequency among patients than in the control (44.6% vs 39.1%, R.R. = 1.25).

Twenty-three patients were also investigated using a DR_β probe after digestion with *Bam* HI and *Taq* I. As revealed in serology, bands corresponding to DR9 and DRw53 were frequently observed, whereas the band for DR2 rarely appeared. For example, 3.8 kb (DR9) and 14.0 kb (DRw53) of *Taq* I and 4.5 kb (DRw53) of *Bam* HI had increased frequencies of 56.5%, 91.3%, and 91.4%, respectively. DR2, however, represented by 12.0 kb, 2.4 kb, 1.9 kb, and 1.7 kb of *Taq* I fragments, showed only a 4.4% frequency in IDDM patients.

Discussion

To investigate the genetic factors related to IDDM in Japanese, especially the HLA-DQ antigen association with IDDM, 56 patients were HLA typed by the microlymphocytotoxicity test, and RFLP analysis was performed by using DQ and DR probes after digestion with *Bam* HI and *Taq* I.

By HLA typing, the important results of increased frequencies for Bw54, Cw1, DR4, DR9, and DRw53 and decreased frequencies for Bw52 and DR2 were observed (Wakisaka et al. 1976, Okimoto et al. 1978, Moriuchi et al. 1980). In reconfirming the previous results, many differences in DQ-antigen distribution were actually demonstrated in this study, in relation to the increased frequencies of DQw9 and DQw4 and decreased frequencies of DQw1. Among all the antigens tested, DQw4 was the unique antigen with the highest difference and relative risk. Since in Japanese patients Bw54, Cw1, and DR4 were in strong linkage disequilibrium with DQw4, DR9 with DQw9, and DRw53 with either DQw4 or DQw9, all these antigens were found to have a high IDDM association in Japanese. It was thought that the primary association might be caused by DQ antigens and the remainder be due to secondary associations. In a similar manner, Bw52 and DR2 were in linkage disequilibrium with DQw1, which might be important in determining IDDM resistance.

These results were reconfirmed by RFLP analysis, where a stronger IDDM association in Japanese showed if the DNA was hybridized with a DQ probe rather than with a DR probe, since the highest and most important relative values were observed by 11.5 kb DQ_β and/or 8.2 kb DQ_α *Bam* HI fragments. These were actually associated with DQw4 and DQw9, showing a relative risk of 3.46 and 2.81, respectively. A 9.2 kb DQ_β *Bam* HI fragment and 6.7 kb DQ_α *Taq* I fragment, which were in relation to DQw1 associated to DR2 (DQw6 for DQ_β antigen), had significantly decreased frequencies among the patients showing the lowest relative risk of 0.11 and 0.07, respectively. The 11.5 kb band might correspond to the positive 12 kb band associated with IDDM in Caucasians (Owerbach et al. 1984). Although increased frequencies of DR9 and DRw53 were found when using a DR_β probe, they were not significantly different when compared to the highest DQ-marker association observed within this study, as mentioned above. Due to the DR-phenotype-matched control used in the RFLP study, the difference was lower than in serology. Our findings, however, support the notion that DQ antigens are the most important antigens in the development of IDDM in Japanese and support similar findings in Caucasians (Cohen-Haguenauer et al. 1985, Tosi et al. 1986, Festenstein et al. 1986).

The suggestion of Todd and co-workers (1987) that the amino acid sequence at the 57th position of the DQ_β chain determines both susceptibility and resistance to IDDM in Caucasians might be different for Japanese. Their observations indicate that 90% of Caucasian IDDM patients are homozygous for the non-Asp-57 *DQ* allele and the remaining 10% are heterozygous, while none of the patients are homozygous for the Asp-57 *DQ* allele. They thus conclude that at least one (usually homozygous)

Table 2. Comparison between IDDM and selected control frequencies and their specificities

Fragment	kb	Specificity	IDDM patients	Matched control	R.R.	*P* value
Bam HI						
DQ_α	24.0	DQw1(DRw6)*	11/47 (23.4%)	3/24 (12.5%)	2.14	
	11.0	DQw1(DR1/2)	13/47 (27.7%)	9/24 (37.5%)	0.64	
	8.2	DQw9+DQw4	42/47 (89.4%)	17/24 (70.8%)	3.46	
DQ_β	11.5	DQw9+DQw4	42/47 (89.4%)	15/20 (75.0%)	2.80	
	9.2	DQw6	2/47 (4.3%)	7/24 (29.2%)	0.11	<0.01
	6.0	DQw5	10/47 (21.3%)	3/24 (12.5%)	1.89	
	3.4	DQw5	10/47 (21.3%)	3/24 (12.5%)	1.89	
DR_β	4.3	DR1	2/23 (8.7%)	-		
	5.3	DR1	2/22 (9.1%)	-		
	12.0	DRw6	5/23 (21.7%)	-		
	5.6	Unknown	11/23 (47.8%)	12/19 (63.2%)	0.53	
	11.9	DRw53	17/23 (73.9%)	-		
	6.8	DRw53	21/23 (91.3%)	17/20 (85.0%)	1.85	
	4.9	DRw53	21/23 (91.3%)	-		
	4.5	DRw53	21/23 (91.3%)	-		
Taq I						
DQ_α	2.7	DQw1(DR1)	14/56 (25.0%)	4/24 (16.7%)	1.67	
	6.7	DQw1(DR2)	2/56 (3.6%)	8/24 (33.3%)	0.07	<0.001
	6.4	DRw52	19/56 (33.9%)	8/24 (33.3%)	1.03	
	6.0	DRw53	50/56 (89.3%)	19/23 (82.6%)	1.75	
	5.7	Unknown	3/56 (5.4%)	3/24 (12.5%)	0.40	
DQ_β	2.8	DQw6	2/56 (3.6%)	5/24 (20.8%)	0.14	<0.05
	5.4	DQw5	26/56 (46.4%)	9/23 (39.1%)	1.35	
DR_β	12.0	DR2	1/23 (4.3%)	11/24 (45.8%)	0.05	<0.005
	2.4	DR2	1/23 (4.3%)	8/24 (33.3%)	0.09	<0.05
	1.9	DR2	1/23 (4.3%)	8/24 (33.3%)	0.09	<0.05
	1.7	DR2	1/23 (4.3%)	8/24 (33.3%)	0.09	<0.05
	10.0	DRw6	5/23 (21.7%)	8/24 (33.3%)	0.56	
	6.8	DRw6	4/23 (17.4%)	11/24 (45.8%)	0.25	
	5.2	DR4	11/23 (47.8%)	15/24 (62.5%)	0.55	
	9.0	DR5	6/22 (27.3%)	5/23 (21.7%)	1.35	
	3.8	DR9	13/23 (56.5%)	10/19 (52.6%)	1.17	
	14.0	DRw53	21/23 (91.3%)	19/24 (79.2%)	2.76	
	2.9	DRw53	21/23 (91.3%)	19/24 (79.2%)	2.76	
	5.8	DRw53	21/23 (91.3%)	19/24 (79.2%)	2.76	

* DR antigen in parentheses indicates the linkage disequilibrium to DQ antigen because DQ_α alleles are not yet specified within the new nomenclature and because of their known linkage disequilibrium to DR_β alleles

non-Asp-57 DQ_β allele is necessary for the development of IDDM (Todd et al. 1987).

However, this may be different in the case of Japanese because amino acid sequences of the β chains of both DQw4 and DQw9 from healthy Japanese, which are strongly associated with IDDM, have already been determined, and their 57th residue is known to be aspartic acid (Gregersen et al. 1986, Todd et al. 1987). This is summarized in Table 3, where the amino acid residue of the 57th position of both DR and DQ chains from different haplotypes are shown (Silver 1986, Bell et al. 1987) in relation to IDDM susceptibility between the two ethnic groups.

Since 24% of the patients in our data were *DR4.1-DQw4* and *DR9-DQw9* heterozygotes, at least 24% of Japanese IDDM patients might be homozygous for *Asp-57* of the DQ_β chain, if there are no DQ_β sequence changes between patients and healthy Japanese. Moreover, only DQw1.1(DQw5) and DQw1.19(DQw6) are known to be non-Asp-57 *DQ* alleles among Japanese cases, where more than 60% of Japanese IDDM patients are supposed to be homozygous for *Asp-57*. The frequency might change if another non-Asp-57 DQ_β allele is found, and great differences may become apparent between Japanese and Caucasians patients.

Table 3. Amino acid residue of the 57th position and association to IDDM

Haplotype	Predisposition to IDDM	Ethnic group	57-DR_β	57-DQ_β
*DR1-DQw5(DQw1.1)**	susceptible	Jap./Cau.†	Asp	Val
DR2-DQw5(DQw1.AZH)	susceptible	Caucasian	Asp	Ser
DR3-DQw2	susceptible	Caucasian	Asp	Ala
DR4.3-DQw8(DQw3.2)	susceptible	Caucasian	Asp	Ala
DRw6-DQw6(DQw1.19)	susceptible	Jap./Cau.	Asp	Ala
DR4.1-DQw4(DQWa)	susceptible	Japanese	Ser	Asp
DR9-DQw9(DQw3.3)	susceptible	Japanese	Val	Asp
DR2-DQw6(DQw1.2)	resistant	Caucasian	Asp	Asp
DR2-DQw6(DQw1.12)	resistant	Japanese	Asp	Asp
DR4.2-DQw7(DQw3.1)	resistant	Jap./Cau.	Asp	Asp
DR5-DQw7(DQw3.1)	resistant	Jap./Cau.	Asp	Asp

* Previous designation
† Jap. (Japanese)/Cau. (Caucasian)

To explain this discrepancy, two explanations might be possible. One is that the Asp-57 idea applies only for Caucasians, due to a different residue of the DQ_β chain, which must be critical for IDDM susceptibility in Japanese. The second, perhaps more attractive possibility is that the 57th residue of the $DQw4_\beta$ or $DQw9_\beta$ chain from Japanese patients might be different from that of healthy cases, that is, the 57th residue of DQw4 and DQw9 from patients might be nonaspartic acid, while that from healthy cases might be aspartic acid. Interestingly, although the 57th residue of DR_β chain is usually aspartic acid, both DR4.1 and DR9 have an amino acid other than aspartic acid in this particular residue. Since the amino acid at the 57th position is highly conserved among the class II antigens, a gene conversion-like event has been thought to occur between DR4.1 and DQw4 or between DR9 and DQw9, thereby producing non-Asp-57 DQ antigens. To clarify which possibility might be true and what mechanism might take place in relation to IDDM in Japanese, we have further work in progress.

Acknowledgments. The authors are very grateful to Mr. K. Masuda, Ms. M. Keira, and Ms. H. Watabe for their excellent technical assistance. This work was supported in part by the Research Grant for the Intractable Diseases from the Ministry of Health and Welfare of Japan, and by a grant-in-aid for scientific research (No. 62570210) from the Ministry of Education, Science, and Culture, Japan.

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Received March 17, 1988; received revised version June 16, 1988