Association of HLA-DR Phenotypes and T-Lymphocyte–Receptor β-Chain–Region RFLP With IDDM in Japanese

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Fifty Japanese patients with insulin-dependent diabetes mellitus (IDDM) and 94 normal subjects were genotyped for *Bgl*II restriction-fragment–length polymorphism (RFLP) of the T-lymphocyte–receptor β-chain (TLRβ)–region gene and analyzed in relation to HLA-DR phenotypes. The antigen frequencies of DR4 and DR9 in the IDDM population were significantly higher than those in the normal population, with relative risks of 1.87 ($P < .02$) and 2.42 ($P < .01$), respectively. Hybridization of digested DNA with the TLRβ probe revealed two alleles of 9.3 and 8.6 kilobases (kb). The allele frequency of 8.6 kb in patients with IDDM (79%) was significantly ($P < .05$) higher than that in normal subjects (64%). When TLRβ-region RFLP in IDDM was further analyzed with respect to the HLA-DR phenotypes, the frequency of 8.6 kb was significantly increased in patients with DR4 but not DR9 (DR4/X) and those with DR9 but not DR4 (DR9/X) compared with the frequency found in normal subjects ($P < .05$); the relative risks of 8.6 kb in DR4/X and DR9/X were 2.77 and 4.98, respectively. Although the frequencies of HLA-DR phenotypes and of TLRβ-region RFLP in IDDM and normal subjects were apparently different from those reported for Caucasians, this population-association study indicates that in the Japanese, genes conferring susceptibility to IDDM exist near or at the HLA-DR and the TLRβ loci, as has been demonstrated in Caucasians. *Diabetes* 37:1633–36, 1988

Autoimmune processes targeting islet cell antigens are supposed to be involved in the pathogenesis of insulin-dependent diabetes mellitus (IDDM; 1). In general, the immune response is initiated with the recognition of an antigen by the T-lymphocyte, which takes place only when the antigen is presented together with major histocompatibility complex (MHC) products on the surface of an antigen-presenting cell (2).

In the last decade, considerable interest has been directed toward the association between IDDM and the HLA system (3,4). A gene that controls susceptibility to IDDM is widely believed to exist on chromosome 6 in the HLA region. However, recent studies indicate that an additional non-HLA-linked gene may contribute to susceptibility to IDDM (5–7).

T-lymphocyte–receptor-recognizing antigens consist of two polypeptide chains, α and β, which are linked by a disulfide bridge (8–10). cDNA corresponding to the murine T-lymphocyte–receptor β-chain (TLRβ) gene has been cloned (11,12), which enabled identification of the TLRβ locus on chromosome 7 (13). Subsequently, based on a cross-hybridization of murine cDNA, restriction-fragment–length polymorphism (RFLP) sites produced by a restriction endonuclease *Bgl*II within and between the constant regions of human TLRβ gene were determined (14). Recently, Hoover et al. (15) demonstrated that the frequency of certain allelic forms of an RFLP detected with the TLRβ-region gene are increased in Caucasians with IDDM. Certain associations of TLRβ-region RFLP genotypes with HLA-DR phenotypes were also reported.

Caucasians and Japanese are obviously genetically different, and the frequent HLA-DR phenotyes in IDDM differ between Caucasians (DR3 and DR4; 3) and Japanese (DR4 and DR9; 4). Therefore, the frequency of alleles at the TLRβ-region RFLP may also differ between the two ethnic populations. For this reason, we examined the TLRβ-region RFLP with respect to HLA-DR phenotypes in patients with IDDM and normal subjects among Japanese.

MATERIALS AND METHODS

Subjects. Fifty unrelated Japanese patients with IDDM (30 males, 20 females, aged 11–52 yr) and 94 normal con-

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Received for publication 17 February 1988 and accepted in revised form 8 June 1988.

trol subjects (52 males, 42 females, aged 22–46 yr) were studied after informed consent was obtained. The patients were not obese at diagnosis, onset of diabetes was often sudden, and their average age at onset was 24 ± 8 yr (mean ± SD). Diabetic ketoacidosis was commonly found, and insulin therapy was necessary in all patients. The patients and the control subjects were divided into four subgroups according to the HLA-DR phenotypes: patients or control subjects with DR4 but not DR9 (DR4/X), those with DR9 but not DR4 (DR9/X), those with both DR4 and DR9 (DR4/9), and those with neither DR4 nor DR9 (DRX/X). The 94 control subjects studied for TLRβ-region RFLP were selected from healthy adults with known HLA-DR phenotypes so that numbers of the subjects in each of the four HLA-DR subgroups were sufficient for a statistical comparison of RFLP allele frequencies. For comparison of antigen frequencies of DR4 and DR9 in IDDM patients and in the normal population, another group of 478 Japanese healthy adults was used as a more reliable reference.

HLA-DR typing. HLA-DR phenotypes were determined for all patients by a standard microcytotoxicity method (16).

DNA analysis. Genomic DNA was isolated from whole blood as previously described (17). Ten to 15 μg DNA was digested with 2 U *Bgl*II (Boehringer Mannheim, Indianapolis, IN) per microgram DNA for 2 h at 37°C. The digested DNA was electrophoresed on 0.7% agarose gels for 36–48 h and transferred to nitrocellulose filters (Schleicher & Schuell, Keene, NH) via Southern blotting (18). Filters were hybridized with DNA probe labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) by nick translation (19). Hybridization was carried out in 5× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll, and 50 μg/ml denatured salmon sperm DNA for 16 h at 65°C. Southern filters were washed at 65°C for 45 min in 2× SSC and 0.1% sodium dodecyl sulfate (SDS), then for 30 min in 0.2× SSC and 0.1% SDS before autoradiography. The TLRβ probe used was the 0.7-kilobase (kb) *Pst*I-*Eco*RI fragment containing the constant region of human TLRβ gene (Oncor, Gaithersburg, MD; 17).

Statistical analyses. Analyses were performed with the χ^2-test, with Yates' correction if needed.

RESULTS

HLA-DR typing showed that 40 of 50 IDDM patients (80%) were positive for either DR4 or DR9: 18 DR4/X$^+$, 10 DR9/X$^+$, and 12 DR4/9$^+$. The antigen frequencies of DR4 and DR9 in IDDM subjects were significantly higher than those in 478 subjects from the normal population, with relative risks of 1.87 ($P < .02$) and 2.42 ($P < .01$), respectively (Table 1).

TABLE 1
Frequency of HLA-DR antigens in IDDM patients and control subjects

	Control (N = 478)		IDDM (N = 50)		
Antigen	n	AF(%)	n	AF(%)	RR
DR4	213	44.6	30	60.0	1.87*
DR9	117	24.5	22	44.0	2.42†

AF, antigen frequency; RR, relative risk.
*$P < .02$, †$P < .01$.

*Bgl*II digestion of all specimens detected TLRβ–constant-region DNA fragments of 9.3 and 8.6 kb, as previously reported (15).

In 94 control subjects, all the genotype frequencies were in Hardy-Weinberg equilibrium, proving that these subjects can be used as an adequate control sample for further analysis (Table 2). There was no statistically significant difference in the incidence of TLRβ-region RFLP alleles among the HLA-DR subgroups of control subjects. Therefore, all control subjects were taken as one group, regardless of DR phenotypes, unless otherwise stated.

As shown in Table 3, the allele frequency of 8.6 kb was significantly ($P < .05$) higher in patients with IDDM (79%) and also in the IDDM subgroups of DR4/X (83%) and DR9/X (90%) than that in the control group (64%). The relative risks of 8.6 kb in DR4/X and DR9/X groups were 2.77 and 4.98, respectively. The allele frequency of 8.6 kb in IDDM patients with DR4/X (83%) was also significantly ($P < .05$) higher than that in control subjects with DR4/X (62%). Similarly, the allele frequency of 8.6 kb in IDDM patients with DR9/X (90%) was significantly ($P < .05$) higher than in control subjects with DR9/X (64%; Tables 2 and 3). When TLRβ-region RFLP was expressed based on the genotypes, the frequency of homozygous state 8.6/8.6 was significantly ($P < .05$) higher in DR4/X and DR9/X than in control subjects (Table 3). The relative risk of 8.6/8.6 in DR9/X (6.16) was twice as high as that in the DR4/X (3.08) subgroup.

DISCUSSION

Increased frequencies of certain HLA-DR antigen phenotypes in IDDM have been demonstrated in various ethnic groups. In Caucasians, DR3 and DR4 are associated with susceptibility to IDDM (3), and in Japanese patients with

TABLE 2
T-lymphocyte–receptor β-chain (TLRβ) restriction-fragment–length polymorphism in control subjects

	TLRβ allele frequency		TLRβ genotype frequency		
Antigen	9.3 kb	8.6 kb	9.3/9.3	9.3/8.6	8.6/8.6
DR4/X	22/58 (38)	36/58 (62)	4/29 (14)	14/29 (48)	11/29 (38)
DR9/X	21/58 (36)	37/58 (64)	3/29 (10)	15/29 (52)	11/29 (38)
DR4/9	9/20 (45)	11/20 (55)	2/10 (20)	5/10 (50)	3/10 (30)
DRX/X	15/52 (29)	37/52 (71)	1/26 (4)	13/26 (50)	12/26 (46)
Total	67/188 (36)	121/188 (64)	10/94 (11)	47/94 (50)	37/94 (39)

Values in parentheses are percentages. kb, Kilobases.

TABLE 3
Association of T-lymphocyte–receptor β-chain (TLRβ) restriction-fragment–length polymorphism with HLA-DR phenotypes in IDDM patients

	TLRβ allele frequency			TLRβ genotype frequency			
Subjects	9.3 kb	8.6 kb	Relative risk	9.3/9.3	9.3/8.6	8.6/8.6	Relative risk
IDDM	21/100 (21)	79/100 (79)	2.08*	3/50 (6)	15/50 (30)	32/50 (64)	2.74†
DR4/X	6/36 (17)	30/36 (83)	2.77*	0/18 (0)	6/18 (31)	12/18 (69)	3.08*
DR9/X	2/20 (10)	18/20 (90)	4.98*	0/10 (0)	2/10 (20)	8/10 (80)	6.16*
DR4/9	7/24 (29)	17/24 (71)		2/12 (17)	3/12 (25)	7/12 (58)	
DRX/X	6/20 (30)	14/20 (70)		1/10 (10)	4/10 (40)	5/10 (50)	
Control	67/188 (36)	121/188 (64)		10/94 (11)	47/94 (50)	37/94 (39)	

Values in parentheses are percentages. kb, Kilobases.
*$P < .05$, †$P < .01$.

IDDM, the frequencies of DR4 and DR9 are reported to be increased (4). In this study, significantly high relative risks of DR4 and DR9 were found in IDDM patients, confirming that a gene influencing susceptibility to IDDM is located near or at the HLA-DR locus. However, failure to explain the mode of inheritance of susceptibility to IDDM in terms of serologically defined HLA-DR specificities has led to the postulation that there are additional major genetic loci for IDDM that are not linked to the HLA region (5–7).

In this study, we analyzed a TLRβ-region RFLP in 50 patients with IDDM and 94 control subjects to determine whether a second non-HLA-linked locus relating to IDDM is present near or at the TLRβ locus. The allele frequency of 8.6 kb was significantly higher in IDDM patients (79%) than in the control subjects (64%). Hoover et al. (15) found in their study of 105 IDDM and 154 normal subjects that the allele frequency of 8.6 kb was 56% and 49%, respectively. The difference in the allele frequencies between the two studies suggests that RFLP in the TLRβ region varies among ethnic groups. When TLRβ-region RFLP in IDDM was further analyzed with respect to the HLA-DR phenotypes, the allele frequency of 8.6 kb was significantly increased in DR4/X and DR9/X, resulting in relative risks of 2.77 for DR4/X and 4.28 for DR9/X. However, an association of 8.6-kb allele with DR4/9 or DRX/X was not found. In this respect, there are at least two possible hypotheses for TLRβ and HLA involvement in IDDM, which would have different predictions for the DR4/9 and DRX/X groups. If the TLRβ is relatively more important in individuals with less HLA-derived IDDM susceptibility, then the TLRβ effect would probably be less obvious in the DR4/9 group but more obvious in the DR4/X, DR9/X, and DRX/X groups. Alternatively, IDDM susceptibility in most individuals may be determined by some interaction between HLA- and TLRβ-derived susceptibility factors, and individuals with HLA-derived susceptibility factors (i.e. DR4 and/or DR9) may be more likely to show TLRβ effects, although other individuals (DRX/X) may have an etiologically different form of IDDM and no apparent TLRβ effect. To determine which of these hypotheses is correct, larger-scale population-association studies of IDDM patients are required.

Note also that an association of TLRβ-region RFLP with the DR4 allele was demonstrated in Japanese, whereas the association was not found with the DR4 allele in Caucasians (15). These facts may emphasize the difference in the mode of inheritance of susceptibility to IDDM between the two ethnic groups.

The results presented here clearly indicate that IDDM in the Japanese population is associated with TLRβ-region RFLP as in Caucasians. Because it is well accepted that the Japanese population is highly homogeneous, the observed association of TLRβ RFLP with IDDM is considered to reflect linkage between RFLP and disease susceptibility rather than population stratification in the analyzed sample. However, this RFLP involves a noncoding region, because the *Bgl*II restriction site responsible for the 8.6-kb allele is located between the two constant regions (14). The TLRβ-region RFLP therefore does not necessarily indicate the abnormality in the TLRβ gene itself. This result is similar to the case of the insulin gene; i.e., RFLP of the insulin gene has not been demonstrated in relation to IDDM susceptibility, despite a persistent population association between IDDM and DNA variations found in the upstream of the insulin gene (20,21). Therefore, this result should be interpreted to indicate that the TLRβ-region RFLP is linked to a locus for the susceptibility to IDDM, which is near the TLRβ gene, although a possibility that the susceptibility locus involves the TLRβ gene cannot be excluded.

Although we focus on the HLA-DR gene in this study, associations between IDDM and genes that are in linkage disequilibrium with the HLA-DR gene have also been investigated with recombinant DNA techniques (22–25). More recently, the HLA-DQ gene has been the object of much speculation in regard to pathogenesis of IDDM. Therefore, the TLRβ RFLP marker, when considered with other RFLP markers (e.g., HLA-DQ gene), might be helpful for predicting high-risk individuals, such as siblings of patients who have IDDM.

ACKNOWLEDGMENTS

We thank Dr. Haruhiko Hasegawa of Nagoya First Red Cross Hospital, Dr. Mitsuro Niinomi of Nagoya National Hospital, Drs. Tsunenori Takano and Takuichiro Imagawa of Chukyo Hospital, Dr. Masashi Tsukada of Higashi Municipal Hospital, and Drs. Seiko Nakayama and Keiko Naito of Nagoya Second Red Cross Hospital for providing whole-blood specimens and clinical data of the patients with IDDM. We also gratefully acknowledge the technical assistance of the staff members of the Histocompatibility Laboratory, Nagoya Second Red Cross Hospital, and Ken-ichi Ohya of Aichi Red

Cross Blood Center and the secretarial assistance of Takayo Izuhara.

This study was partly supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture and by grants from the Ministry of Health and Welfare of Japan.

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