The HLA-DR and DQ Genes Control the Autoimmune Response to DNA Topoisomerase I in Systemic Sclerosis (Scleroderma)

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Abstract

HLA class II alleles were determined using the PCR-RFLP method in Japanese systemic sclerosis (scleroderma) patients with ($n = 28$) or without ($n = 34$) anti-topoisomerase I antibodies (anti-topo I). Either the DQB1*0601 or *0301 allele was recognized in all anti-topo I positive patients, compared with 44% of anti-topo I negative patients ($P < 0.00001$, relative risk [RR] > 41) or 58% of Japanese healthy control subjects ($P < 0.00001$, RR > 24). Tyrosine at position 26 in the second hypervariable region in the β1 domain of the DQB1 gene is common to these two alleles and is not present in any other known DQB1 alleles. We also examined immunoreactivities of anti-topo I positive sera to four different autoantigenic B cell epitopes of topo I molecule that were expressed as recombinant fusion proteins. One major B cell epitope, located within the region corresponding to amino acid residues 74–248, was perfectly associated with the amino acid sequence FLEDR at positions 67–71 in the β1 domain of the DRB gene. Two other epitopes, corresponding to 316–441 or 658–700, were associated with the serologically defined HLA-DR52 antigen. Patients with both FLEDR and DR52 demonstrated higher anti-topo I antibody titers. These results suggest that the HLA-DR and DQ genes together control the autoimmune response to topo I in systemic sclerosis. (*J. Clin. Invest.* 1993. 92:1296–1301.) Key words: immunogenetics • PCR-RFLP method • shared epitope • immune response gene • immune suppression gene

Introduction

Systemic sclerosis (scleroderma; SSc)[1] is an autoimmune disease characterized by Raynaud's phenomenon and thickening of the skin. The clinical presentation, however, is highly variable with respect to cutaneous and internal organ involvement (1). Antinuclear antibodies (ANA) are frequently found in sera from SSc patients (1, 2), and it has been established that some types of ANA are closely related to the clinical subsets of SSc. The major ANA in SSc sera include antibodies to centromere and to DNA topoisomerase I (topo I). Anticentromere antibody is detected primarily in patients with limited cutaneous SSc with low frequencies of serious internal organ involvement (1–7), whereas anti-topo I antibody identifies a subset of patients with more extensive skin involvement and pulmonary interstitial fibrosis (1, 6, 7).

The molecular mechanism underlying the development of SSc is unknown, but genetic factors are probably implicated in its pathogenesis. Several immunogenetic studies of SSc patients have been performed and associations with certain HLA class I (8–13), class II (12, 14–21), and class III (13, 20) antigens were reported. However, these HLA associations were not strong and were sometimes inconsistent with each other. In some studies, no significant association was found (22–24). We suspect that the associations in these studies may be obscured by the lack of consideration of the concept that SSc is a widely varying disease. The serum SSc-specific ANA are useful indices in the clinical field and associate with certain HLA class II antigens (6, 21, 24, 25). Since the HLA class II molecule presents processed antigen to the T cell receptor and results in an antigen-specific immune response (26–28), an association of HLA class II antigens with certain ANA may provide important insight regarding the autoimmune response as well as the pathogenesis of SSc.

We developed recently the PCR–RFLP method, based on digestion of PCR-amplified DNA with allele-specific restriction enzymes as a simple, reliable, and practical technique for HLA class II genotyping (29–33). We have also identified four different autoantigenic B cell epitopes of topo I molecule using nonoverlapping topo I fragments expressed as recombinant fusion proteins and found clinical and immunogenetic associations with these B cell epitopes (34). To clarify the role of HLA class II genes in anti-topo I antibody production in SSc, we adopted, in this study, the PCR-RFLP method for genotyping of the HLA-DRB1, DRB5, DQB1 and DPB1 genes in 62 SSc patients with or without anti-topo I and evaluated an autoimmune response to topo I by examining its association with the autoantigenic B cell epitopes.

Methods

Patients and controls. We selected 62 unrelated Japanese SSc patients who visited the Department of Medicine, Keio University School of Medicine (Tokyo, Japan) for follow-up evaluation during the period between January and July, 1992. All patients fulfilled the American Rheumatism Association preliminary classification criteria for SSc (35). Anti-topo I antibody in the serum from each patient was determined by double immunodiffusion (method described below). All 28 anti-topo I positive patients seen during the 7-mo period and 34 of 68 patients without anti-topo I seen during the same period were included

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Received for publication 14 December 1992 and in revised form 8 March 1993.

1. *Abbreviations used in this paper:* ANA, antinuclear antibody; ER, epitope region; Pcorr, corrected P; RR, relative risk; SSc, systemic sclerosis; topo I, DNA topoisomerase I.

J. Clin. Invest.

0021-9738/93/09/1296/06 $2.00
Volume 92, September 1993, 1296–1301

in this study. No patients with rapidly progressive disease were included.

Clinical information was obtained from all 62 SSc patients. The criteria for disease classification and organ involvement were based on those described elsewhere (34). There were no significant differences in the demographic features between anti-topo I positive and negative patients. Anti-topo I positive patients had higher frequencies of diffuse cutaneous involvement, peripheral vascular disease, and pulmonary interstitial fibrosis than did anti-topo I negative patients (57 vs. 18%, $P < 0.0001$; 43 vs. 9%, $P < 0.0005$; and 82 vs. 26%, $P < 0.0001$, respectively).

Healthy control subjects included 43 Japanese volunteers living in the Tokyo area.

Serological analyses. Serum samples were obtained from all SSc patients at their first visit and stored at −20°C. We detected serum anti-topo I antibody by double immunodiffusion using rabbit thymus extract as an antigen source (36). The anti-topo I antibody titer was defined as the highest serum dilution that clearly formed a precipitin line.

Reactivities to recombinant fusion proteins expressing four different topo I epitope regions (ER) were analyzed by immunoblotting (34). We used four cDNA clones that encoded the autoantigenic B cell epitopes on topo I; ER1, ER2, ER3, and ER4 containing amino acid residues 74–248, 316–441, 485–601, and 658–700, respectively, of the 765 amino acid topo I molecule.

Serologic HLA class II typing. Serologic HLA typing of the HLA-DR and DQ antigens was performed using the standard complement-dependent microlymphocytotoxicity test (37).

PCR-RFLP method. Genomic DNA was isolated by phenol extraction of SDS-lysed and proteinase K–treated peripheral blood leukocytes (38) and then amplified by the PCR procedure using an automated PCR thermal cycler (Perkin Elmer-Cetus, Norwalk, CT). The primers used for specific amplification of the polymorphic exon 2 domains of the DRB1, DRB5, DQB1, and DPB1 genes were previously described (29–33). Amplified DNA was digested by allele-specific restriction endonucleases and subjected to electrophoresis using a 12% polyacrylamide gel. Digested fragments were detected by staining with ethidium bromide, and HLA genotypes were determined on the basis of the RFLP patterns generated as described (29–33).

Statistical analyses. Differences in frequency and mean value between patient groups were tested by Fisher's two-tailed exact test and Student's t test, respectively. Significant P values were corrected (Pcorr) by multiplying by the number of comparisons made. Relative risk (RR) was calculated as the odds ratio. Comparisons of antibody titer between patient groups were made by Wilcoxon rank sum test.

Results

Serologic typing of HLA-DR and DQ. Serologic typing of HLA-DR and DQ antigens was performed for 28 anti-topo I positive, 34 anti-topo I negative SSc patients, and 43 control subjects. DR2 was present in 22 (79%) of 28 anti-topo I positive SSc patients, compared with 32% of anti-topo I negative SSc patients (Pcorr < 0.0005, RR = 7.7) or 33% of control subjects (Pcorr < 0.0001, RR = 7.6). In contrast, DR6 was present in 4% of SSc patients with anti-topo I and less common than in those without anti-topo I (32%, Pcorr < 0.05) or in the control subjects (33%, Pcorr < 0.05). There was no significant difference in the frequency of any other HLA-DR or DQ antigens among these three groups.

Genotyping of HLA-DRB1, DRB5, DQB1, and DPB1 alleles. We determined further the genotypes of HLA class II alleles by the PCR-RFLP method and listed their allelic frequencies among the three groups in Table I. The DRB1*1502-DRB5*0102 haplotype was present in 21 (75%) of 28 SSc patients with anti-topo I, compared with 21% of SSc patients

Table I. The Genotype Frequencies (%) of the HLA-DRB1, DRB5, DQB1, and DPB1 Genes in 62 Japanese Systemic Sclerosis (SSc) Patients with or without Serum Anti-topoisomerase I Antibodies (anti-topo I) and in 43 Japanese Control Subjects

Allele	SSc with anti-topo I (n = 28)	SSc without anti-topo I (n = 34)	Control subjects (n = 43)
DRB1*0101(DR1)	4	12	12
1501(DR2)	4	12	12
1502(DR2)	75*	21	21
1602(DR2)	4	3	0
0401(DR4)	4	3	2
0403(DR4)	11	3	9
0405(DR4)	7	21	33
0406(DR4)	7	6	9
0407(DR4)	4	0	0
0410(DR4)	4	3	0
1101(DR5)	7	9	5
1102(DR5)	0	0	2
1201(DR5)	11	3	2
1202(DR5)	4	0	5
1301(DR6)	0	6	0
1302(DR6)	0	15	14
1401(DR6)	4	3	12
1402(DR6)	0	0	7
1403(DR6)	0	3	0
1405(DR6)	0	6	0
0802(DR8)	7	18	2
0803(DR8)	18	6	19
0901(DR9)	18	18	26
1001(DR10)	0	0	5
DRB5*0101	4	12	12
0102	75‡	21	21
02¶	4	3	0
DQB1*0501(DQ1)	7	15	19
0502(DQ1)	0	9	9
0503(DQ1)	4	6	12
0601(DQ1)	75§	27	40
0602 or 0603**(DQ1)	4	12	12
0604(DQ1)	0	18	16
0301(DQ3)	43	18	19
0302(DQ3)	14	29	14
0303(DQ3)	18	29	23
0401(DQ4)	7	12	23
0402(DQ4)	7	3	7
DPB1*0201	25	15	35
0202	0	9	12
0301	29	9	9
0401	0	6	9
0402	7	35	23
0501	46	56	58
0901	68‖	21	16
1301	4	0	2
1401	4	0	0

* Pcorr < 0.005 vs. both SSc without anti-topo I and control subjects. ‡ Pcorr < 0.0005 vs. both SSc without anti-topo I and control subjects. § Pcorr < 0.0005 vs. SSc without anti-topo I and Pcorr < 0.05 vs. control subjects. ‖ Pcorr < 0.001 vs. SSc without anti-topo I and Pcorr < 0.0005 vs. control subjects. ¶ The second exon is identical among DRB5*02 alleles. ** DQB1*0602 and *0603 were not discriminated in this study.

without anti-topo I (RR = 12) or 21% of control subjects (RR = 11).

DQB1*0601 was present in 21 (75%) of 28 anti-topo I positive SSc patients as expected because of its linkage disequilibrium with the DRB1*1502–DRB5*0102 haplotype, compared with 27% of anti-topo I negative SSc patients (RR = 8.3) or 40% of control subjects (RR = 4.6). There was a tendency for DQB1*0301 in anti-topo I positive SSc patients to be more common than in anti-topo I negative SSc patients (RR = 3.5) or in control subjects (RR = 3.3), but these differences did not reach statistical significance after correcting the *P* values. It is noteworthy that all anti-topo I positive SSc patients possessed either DQB1*0601 or *0301 allele. These two DQB1 alleles share several amino acid residues in the hypervariable region in the β1 domain of the DQB1 gene. Table II shows the phenotypic frequencies of these residues in SSc patients with or without anti-topo I and in control subjects. Of these residues, tyrosine at position 26 in the second hypervariable region (Tyr-26) was in the strongest association with anti-topo I positive SSc patients compared with anti-topo I negative SSc patients (RR > 41) or control subjects (RR > 24). Furthermore, because Tyr-26 is not present in any known DQB1 alleles except for DQB1*0601 or *0301, Tyr-26 is likely to be most important in the development of the anti-topo I autoimmune response. The frequency of arginine at position 70 (Arg-70) was significantly increased in anti-topo I positive SSc patients than in control subjects (RR > 6.4), suggesting that Arg-70 may play some role in the anti-topo I autoimmune response.

With respect to the DPB1 allele, DPB1*0901 was present in 68% of 28 SSc patients with anti-topo I, compared with 21% of anti-topo I negative SSc patients (RR = 8.1) or 16% of control subjects (RR = 11). This increased frequency of DPB1*0901 can be explained by the known linkage disequilibrium with the DRB1*1502–DRB5*0102–DQB1*0601 haplotype in a Japanese population (39). However, two patients having the DRB1*1502–DRB5*0102–DQB1*0601 haplotype did not possess the DPB1*0901 allele, implying that the DPB1*0901 allele itself is not responsible for the anti-topo I antibody production.

Table II. The Phenotypic Frequencies (%) of HLA-DQB1 Amino Acid Residues in 62 Japanese Systemic Sclerosis (SSc) Patients with or without Serum Anti-topoisomerase I Antibodies (anti-topo I) and in 43 Japanese Control Subjects

Amino acid residues in HLA-DQB1	SSc with anti-topo I (*n* = 28)	SSc without anti-topo I (*n* = 34)	Control subjects (*n* = 43)
Methionine-15	100	94	98
Tyrosine-26	100*	44	58
Tyrosine-30	100	91	98
Aspartic acid-57	100	82	95
Arginine-70	100‡	88	81
Threonine-77	100	94	98
^{71}TRAELDT^{77}	100	88	91

^{71}TRAELDT^{77}, the sequence Thr, Arg, Ala, Glu, Leu, Asp, Thr at positions 71–77 in the DQB1 β1 domain. *Pcorr < 0.00005 vs. both SSc without anti-topo I and control subjects. ‡Pcorr < 0.05 vs. control subjects.

21 anti-topo I positive patients had DRB1*1502-DRB5*0102-DQB1*0601 haplotype. Three of the remaining seven anti-topo I positive SSc patients possessed either DRB1*1101 or *0802. The DRB5*0102, DRB1*1101, and DRB1*0802 alleles share the amino acid sequence Phe, Leu, Glu, Asp, Arg at positions 67–71 (^{67}FLEDR^{71}) in the third hypervariable region in the β1 domain of the DRB gene. We hypothesized, therefore, that the combination of Tyr-26 in the DQB1 β1 domain and ^{67}FLEDR^{71} in the DRB β1 domain might be more closely associated with the anti-topo I antibody production. This combination was present in 24 (86%) of 28 anti-topo I positive SSc patients, compared with 29% of anti-topo I negative patients ($P < 0.000001$, RR = 14) or 28% of control subjects ($P < 0.000001$, RR = 16). These results suggest that the autoimmune response to topo I in SSc patients requires at least Tyr-26 in the DQB1 β1 domain and is also controlled by the HLA-DRB genes.

Autoantigenic topo I B cell epitopes and HLA-DR genes. We have reported that anti-topo I positive sera from SSc patients include autoantibodies directed to at least four different ER on topo I molecule (34). Of 28 anti-topo I positive sera in the present study, 24 (86%), 12 (43%), 25 (89%), and 9 (32%) sera reacted with ER1, ER2, ER3, and ER4, respectively. Reactivity with both ER1 and ER3 was found in 21 (75%), of which 13 also reacted to ER2 and/or ER4. There were no significant associations between these 4 ER reactivities and HLA-DQ specificities. When we compared the frequencies of the ^{67}FLEDR^{71} sequence among patients with 4 ER reactivities, ER1 was completely associated with the ^{67}FLEDR^{71} sequence (Table III). Interestingly, all four patients negative for ^{67}FLEDR^{71} had reactivity to only ER3 but not to any other ER. Genotyping of DRB1 for these four patients revealed DRB1*0403/*0405, DRB1*0403 homozygote, DRB1*0405/*0406, and DRB1 *0406/*1202. The DRB1*0403 or *0406 allele was commonly observed among these four patients. Moreover, we found another four ^{67}FLEDR^{71} positive patients with DR4-related alleles, all of whom had ER3 reactivity. Next we examined the associations of 4 ER reactivities and the serologically defined HLA-DR52 antigen in the 24 ^{67}FLEDR^{71} positive SSc patients with anti-topo I (Table III). Significant association of the DR52 antigen with ER2 or ER4 reactivity was apparent. These results suggest that (*a*) ER1 reactivity is strongly asso-

Table III. Frequencies of the ^{67}FLEDR^{71} Sequence (Phe, Leu, Glu, Asp, Arg at Positions 67–71) in the HLA-DRB1 or HLA-DRB5 β_1 Domain and the HLA-DR52 Antigen in 28 Japanese Systemic Sclerosis Patients with Serum Anti-DNA Topoisomerase I Antibody According to the Reactivity to Four Different ER of DNA Topoisomerase I Molecule

	ER1* (*n* = 24)	ER2 (*n* = 12)	ER3 (*n* = 25)	ER4 (*n* = 9)
^{67}FLEDR^{71} positive	24 (100%)‡	12 (100%)	21 (84%)	9 (100%)
DR52 positive	12 (50%)	10 (83%)§	10 (48%)	8 (89%)§
DR52 negative	12 (50%)	2 (17%)	11 (52%)	1 (11%)
^{67}FLEDR^{71} negative	0	0	4 (16%)	0

Values are the number (%). *ER1, ER2, ER3, and ER4 contain amino acid residues 74–248, 316–441, 485–601, and 658–700 of the DNA topoisomerase I molecule, respectively. ‡P < 0.0001 vs. ^{67}FLEDR^{71} negative. §P < 0.005 vs. DR52 negative.

ciated with the ^{67}FLEDR^{71} sequence; (*b*) ER2 or ER4 reactivity requires both the ^{67}FLEDR^{71} sequence and the DR52 antigen; and (*c*) ER3 reactivity is probably associated with some of the DR4 related alleles. Of 12 patients having both the ^{67}FLEDR^{71} sequence and the DR52 antigen, 6 were subtyped as DRB1*0802 or *0803 and lacked the DRB3 gene. Then, the association of the serologically defined DR52 antigen may depend on the sequence Glu, Tyr, Ser, Thr, comprising positions 9–12 in the first hypervariable region in the β1 domain of the DRB1 gene, which is commonly shared among DR52-associated antigens (DR3, DR5, DR6, and DR8).

Anti-topo I antibody titer and HLA-DR genes. The anti-topo I antibody titer was compared among 28 anti-topo I positive SSc patients with the ^{67}FLEDR^{71} sequence in their DRB β1 domain or the DR52 antigen (Fig. 1). The anti-topo I antibody titer was significantly higher in ^{67}FLEDR^{71} positive patients than in those negative for this sequence. Furthermore, among 24 ^{67}FLEDR^{71} positive patients the antibody titer was significantly higher in those with the DR52 antigen than in those without this antigen, suggesting that ^{67}FLEDR^{71} and the DR52 antigen participate in the regulation of the immune response as reflected by the antibody titer.

Discussion

In this study, molecular genetic analysis of HLA class II genes was performed in 28 Japanese SSc patients with anti-topo I antibody. The new findings show that: (*a*) anti-topo I antibody production was associated with both Tyr-26 in the DQB1 β1 domain and the ^{67}FLEDR^{71} sequence in the DRB1 or DRB5 β1 domain; (*b*) immunoreactivity to the topo I autoantigenic B cell epitopes was associated with the ^{67}FLEDR^{71} sequence and the serologically defined DR52 antigen; and (*c*) anti-topo I antibody titer was associated with the ^{67}FLEDR^{71} sequence and DR52. Therefore, it is likely that a combination of the HLA-DRB and DQB1 genes control the anti-topo I antibody production and that this production is associated with the shared epitopes common to the β1 domain of several HLA class II genes rather than with a single HLA class II gene.

Reveille et al. (40) showed that the broad specificity HLA-DQ3 (DQB1*0301, *0302, and *0303) was most strongly associated with the anti-topo I response in Caucasian and American black SSc patients, and the remaining DQ3-negative patients had DQB1*0601, *0602, *0604, or *0402. Therefore, they concluded that tyrosine at position 30 (Tyr-30) and Thr, Arg, Ala, Glu, Leu, Asp, Thr comprising positions 71–77 (^{71}TRAELDT^{77}) in the DQB1 β1 domain were associated with the anti-topo I response. Although no statistically significant differences were obtained in the phenotypic frequencies (Table II) as well as in the genotypic frequencies (data not shown) of Tyr-30 and ^{71}TRAELDT^{77} in this study, all Japanese anti-topo I positive patients had both Tyr-30 and ^{71}TRAELDT^{77}. Therefore, our observation supports, in part, their hypothesis that these amino acid residues form the minimum requirement for the anti-topo I response. However, they found no HLA-DRB sequence associated with anti-topo I. The discrepancy between our results and their observations may be explained by the differences in geographic environmental factors or by the concern of "founder effect" because the Japanese have historically been a genetic isolate. However, we argue that if an HLA-disease association is postulated to be observed worldwide among many ethnic groups, the anti-topo I response is more closely associated with Tyr-26 than Tyr-30 or ^{71}TRAELDT^{77} because of the following reasons: (*a*) Tyr-26 was found in all Japanese patients with anti-topo I as shown in the present study; (*b*) DQB1*0301 was most frequently found in Caucasians and American blacks with anti-topo I in Reveille's report (57 and 82%, respectively) (40); (*c*) all five native American Choctaw SSc patients with anti-topo I were shown to have DQB1*0301 (41); and (*d*) Morel et al. (42) noted that 87% of American Caucasian SSc patients with anti-topo I had the DR11-related alleles, almost all of which is in linkage disequilibrium with DQB1*0301.

DRB1*1104, possessing the ^{67}FLEDR^{71} sequence and in linkage disequilibrium with DQB1*0301, was reported to be present in 63% of 30 American Caucasian SSc patients with anti-topo I (42), but was not found in 28 Japanese SSc patients with anti-topo I in this study. DRB1*1104 is observed frequently in Caucasian and black populations, whereas it is not present in a Japanese population (43). In contrast, the DRB1*1502-DRB5*0102-DQB1*0601 haplotype is second most frequent in a Japanese population but is quite rare in Caucasian populations (43). Therefore, HLA class II alleles associated with anti-topo I antibody appear to depend on the distribution of the regional HLA class II alleles in normal population. These insights may explain the discrepancy among previous reports describing that anti-topo I was associated with DR5 in Caucasians (6, 25) and with DR2 in Japanese (34).

Autoantigenic B cell epitopes on topo I molecule were analyzed by several investigator groups (34, 44–47). The location of ER3 was consistent with that of major B cell epitope determined by previous studies (45, 46). In this study, we showed that each of four different B cell epitopes was associated with the different HLA-DR genes, suggesting that the specific immune response to each B cell epitope is controlled by different T cell epitopes. To confirm this hypothesis, further analyses of T cell proliferation responses to each B cell epitope will be necessary.

It is well known that the immune response to an antigen is controlled by both immune response and suppression genes (48–50). Sasazuki and his co-workers proposed a model that the HLA-DR and DQ molecules were the products of immune response and suppression genes, respectively, based on their studies of the T cell responses to schistosoma japonicum anti-

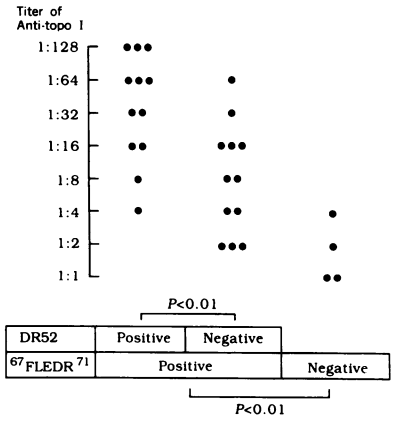

Figure 1. Titer of anti-topo I antibodies in sera from 28 Japanese SSc patients according to the presence of the sequence Phe, Leu, Glu, Asp, Arg at positions 67–71 (^{67}FLEDR^{71}) of the DRB1 or DRB5 β1 domain and the DR52 antigen. The serum antibody titer was determined by double immunodiffusion, the ^{67}FLEDR^{71} sequence was determined by DNA typing using the PCR-RFLP method, and the DR52 antigen was determined by serological typing. See methodological details in Methods. *P* values at the bottom of the columns were calculated by Wilcoxon rank sum test.

gen (51) and streptococcal cell wall antigen (52). Application of this model on the anti-topo I antibody production leads us to the speculation that the absence of Tyr-26 in the DQB1 β1 domain might, as the immune suppression gene, lead to nonresponsiveness against topo I antigen and the ^{67}FLEDR^{71} sequence in the DRB β1 domain and the DR52 antigen might, as the immune response gene, control the intensity of the autoimmune response.

Tyrosine at position 26 in the DQB1 β1 domain was also shown to be one of the amino acid residues associated with another SSc-specific autoantibody, anticentromere (53). Thus, it appears that the putative antigen binding cleft at position 26 in the DQB1 β1 outermost domain is important for these two autoantibody production, and different HLA-DR genes define the reactivity to the two different antigens.

Acknowledgments

We express our thanks to David B. Stollar (Tufts University School of Medicine, Medford, MA) for his critical reading of this manuscript and to Shuichi Tsuji (Keio University School of Medicine, Tokyo, Japan) for arrangement of our project of the HLA-ANA association.

This work was supported by the Scleroderma Grant for Intractable Disease from the Japanese Ministry of Health and Welfare.

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