Genetic Analysis of Dilated Cardiomyopathy

— HLA and immunoglobulin genes may confer susceptibility —

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To identify genetic factors in the immune system which control the susceptibility to dilated cardiomyopathy (DCM), HLA class II DNA typing was performed in 61 Japanese patients, using PCR/SSO probe analyses. The frequencies of HLA-DQB1*0503 (15% vs 5%; RR=3.06, $\chi^2=7.19$) and DQB1*0604 (21% vs 10%; RR=2.41, $\chi^2=6.20$) were significantly increased and that of HLA-DQB1*0502 (RR=1.74) was slightly increased in the DCM patients. The frequency of DQB1*0303 (16% vs 31%; RR=0.44, $\chi^2=5.16$) was significantly decreased in the patients. The increased HLA-DQB1 alleles have a histidine residue in common at the 30th codon for the HLA-DQ β chain. Among the genetic markers studied by Southern blot analyses, IGLV (immunoglobulin lambda light chain, pV3.3) showed a strong association with DCM, i.e. A2/A2 genotype was found in 37.7% of patients whereas it was observed in only 18.9% of the control subjects (RR=2.6, $\chi^2=7.77$). The frequency of this genotype was higher in patients under age 45 years at the time of diagnosis (45.5%, RR=3.6, $\chi^2=10.02$). These results suggest that HLA and immunoglobulin genes are closely linked to susceptibility to DCM.

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Key words:

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DILATED cardiomyopathy (DCM), a cardiac disorder of unknown etiology is characterized by ventricular dilatation, decreased myocardial contractility and a poor prognosis. Genetic related factors have long

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TABLE I SEQUENCE SPECIFIC OLIGONUCLEOTIDES PROBES FOR THE DQA1 GENE

SSO	Sequence 5' to 3'	Specificity*	A.A.**
2501	TGGCCAGTACACCCATGA	0101,0102,0401,0501.	22-28
2502	TGGCCAGTTCACCCATGA	0103,0201,0601.	22-28
2503	TGGGCAGTACAGCCATGA	0301,0302.	22-28
3401	GAGATGAGGAGTTCTACG	0101.	31-37
3402	GAGATGAGCAGTTCTACG	0102,0103,0501.	31-37
3403	GAGACGAGCAGTTCTACG	0401,0601	31-37
4102	ACCTGGAGAGGAAGGAGA	0103.	38-44
5501	TCAGCAAATTTGGAGGTT	0101,0102,0103.	51-57
5502	TCCACAGACTTAGATTTG	0201.	51-57
5503	TCCGCAGATTAGAAAGAT	0301,0302.	51-57
5504	TCAGACAATTTAGATTTG	0401,0501,0601.	51-57
6901	ATGGCTGTGGCAAAACAC	0101,0102,0103.	66-71
6902	ATCGCTGTGCTAAAACAT	0201,0301,0302	66-71
6903	ATCGCTGTCTAAAACAT	0301,0501.	66-71
6904	ATCGCTGTGACAAAACAC	0401,0601.	66-71
7502	CTTGAACATCCTGATTAA	0201,0401,0601.	72-78
7504	CTTGAACAGTCTGATTAA	0501.	72-78

*The allele designations are those of the official nomenclature committee (WHO Nomenclature Committee 1991).

**Corresponding amino acid position.

TABLE II SEQUENCE SPECIFIC OLIGONUCLEOTIDES PROBES FOR THE DQB1 GENE

SSO	Sequence 5' to 3'	Specificity*	A.A.**
2301	GACCGAGCTCGTGCGGGG	0401.	20-26
2302	AACGGGACCGAGCGCGTG	0303,0402.	20-26
2601	CGGGGTGTGACCAGACAC	0501,0502,0503.	25-30
2602	CGTTATGTGACCAGATAC	0601,0301.	25-30
2603	CGTCTTGTGACCAGATAC	0602,0302,0303.	25-30
2604	CGTCTTGTAACCAGACAC	0603,0604.	25-30
2606	CGTCTTGTAACCAGATAC	0605.	25-30
3702	AGGAGGACGTGCGCTTCG	0601.	35-41
4501	GACGTGGAGGTGTACCGG	0301.	43-48
5701	GCGGCCTGTTGCCGAGTA	0501,0604,0605.	54-60
5702	GCGGCCTAGCGCCGAGTA	0502,0504.	54-60
5703	GGCGGCCTGACGCCGAGT	0503,0601.	54-60
5704	GCGGCCTGATGCCGAGTA	0503,0602,0603.	54-60
5705	GGCTGCCTGCCGCCGAGT	0201.	54-60
5706	GGCCGCCTGACGCCGAGT	0301,0303.	54-60
5707	GGCCGCCTGCCGCCGAGT	0302.	54-60
5708	GCGGCTTGACGCCGAGTA	0401,0402.	54-60
7002	GACCCGAGCGGAGTTGGA	0601.	70-76

The same as the footnotes of Table I.

been suspected, because some patients with DCM had a familial onset and some patients were the products of consanguineous marriages. Immunological dysfunction such as the presence of auto-antibodies to the myo-

cardium! and persistent infection with Cox-sackie B virus² have been noted in patients with DCM. The immune response to foreign antigens, e.g. viral antigens is controlled by factors such as genes for HLA antigens, im-

TABLE III ASSOCIATION BETWEEN DCM AND HLA-DQB1 ALLELES

DQB1*	Control n=336	DCM			amino acid		
		Total n=61	Ab (+) n=35	Ab (-) n=24	26	30	38
0501	11.9%	13.1%	11.8%	16.7%	G	H	V
0502	3.9	8.2	2.9	16.7*	G	H	V
0503	5.4	14.8**	14.7#	16.7	G	H	V
0601	39.9	29.5	26.5	29.2	Y	Y	V
0602	12.8	9.8	8.8	8.3	L	Y	A
0603	0.6	0	0	0	L	H	A
0604	10.1	21.3*	26.5##	12.5	L	H	A
0605	1.8	0	0	0	L	Y	A
0201	1.8	0	0	0	L	S	V
0301	21.7	21.3	17.6	20.8	Y	Y	A
0302	15.8	9.8	11.8	8.3	L	Y	A
0303	30.7	16.0*	26.5	12.5	L	Y	A
0401	28.3	31.1	29.4	33.3	G	Y	A
0402	5.7	6.6	8.8	4.2	G	Y	A

The allele designation is that of the official Nomenclature Committee (WHO Nomenclature Committee 1991). The statistical significance of the association between HLA and disease was determined by chi-square analysis with Yates' correction.

* $P < 0.05$, ** $P < 0.01$ # $P < 0.005$, ## $P < 0.0001$

munoglobulins and T cell receptors³⁻⁵ To elucidate the genetic background related to altered immune responses in DCM, DNA typing of HLA class II genes (DQ α and DQ β), restriction fragment length polymorphism (RFLP) analysis of immunoglobulin lambda light chain variable gene (IGLV), and those of T cell receptor α and β genes was carried out.

SUBJECTS AND METHODS

Subjects

The study group consisted of 61 Japanese patients with DCM (43 men and 18 women, ranging in age from 15–69 years; with a mean age of 47 ± 13 years at the present study, 44 ± 15 years at the time of diagnosis). These patients were identified at the following clinical centers of the Idiopathic Cardiomyopathy Research Committee (The Ministry of Welfare and Health of Japan); Kurume University Hospital, Osaka Medical College Hospital, National Cardiovascular Center Hospital, Hokkaido University Hospital, The Jikei University Aoto Hospital, Juntendo University Hospital, Kyoto University Hospital, Yamaguchi University Hospital, Tokyo University Hospital. All

the patients fulfilled the diagnostic criteria of DCM proposed from the Idiopathic Cardiomyopathy Research Committee. Coronary angiography and endomyocardial biopsy were performed in all patients to exclude coronary artery disease. We also excluded those with underlying valvular heart disease, acute myocarditis, hypertrophic cardiomyopathy and other cardiac or systemic diseases involving the heart muscle.

Method

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from peripheral granulocytes of each subject, using standard techniques⁶ Genomic DNA was subjected to PCR in 10 mM Tris-HCl (pH 8.4 at 24 °C), 50 mM KCl, 0.1 mg/ml gelatin, 0.02% NP40 and 2.0 mM MgCl₂ to amplify the second exons of the DQA1 or DQB1 gene, using thermostable DNA polymerase (Ampli Taq, Perkin Elmer Cetus). DQA1 and DQB1 genes were amplified by using AKQAP1 (5'-ATGGTGTAACCTTGACCAGT-3') and AKQAP2 (5'-TTGGTAGCAGCGGTAGAGTTG-3') or AKQBP1 (5'-CATGTGCTACTTCACCAACGG-3') and AKQBP2 (5'-CTGGTAGTTGTGTCTGCACAC-3'),

TABLE IV ASSOCIATION BETWEEN DCM AND HLA-DQA1 ALLELES

DQA1*	Control n=336	DCM n=61
0101	19.0%	26.2%
0102	24.4	31.1
0103	39.6	32.8
0201	0.6	0
0301	68.2	54.1*
0401	2.1	3.3
0501	16.1	14.8
0601	4.2	3.3

The same as the footnotes of Table III.

* $P < 0.05$

respectively. The denaturation, annealing and extension step for the amplification of DQA and DQB gene were each 1 min at 96 °C, 55 °C, and 72 °C, respectively. One microgram of DNA was subjected to 30 cycles of amplification with a thermal cycler (Perkin Elmer Cetus).

Dot blot analysis with sequence specific oligonucleotides (SSOs)

The amplified DNAs were spotted onto a nylon membrane (Hybond N plus, Amersham) and immobilized by alkaline denaturation in 0.4 N NaOH. Filters were prehybridized for 1 h at 54 °C in 10 ml hybridization buffer composed of 50 mM Tris-HCl (pH 8.0), 3.0 M tetramethylammonium chloride, 2 mM EDTA (pH 8.0), 5× Denhardt's solution, 0.1% SDS and 100 µl/ml heat-denatured herring sperm DNA. Sequence specific oligonucleotide probes (SSOPs) (Tables I, II) were labelled with [γ -³²P]-ATP (ICN Biomedicals Inc.), using T4 polynucleotide kinase (TOYOBO Co. Ltd.). After hybridization with labelled probes at 54 °C for 1 h, the filters were washed twice for 5 min each at room temperature in 2× SSPE and 0.1% SDS with constant gentle agitation, once for 10 min at room temperature in TMAC solution [50 mM Tris-HCl (pH 8.0), 3.0 M tetramethylammonium chloride, 2 mM EDTA and 0.1% sodium dodecyl sulfate (SDS)] and twice for 15 min at 58 °C to 60 °C in TMAC solution. The procedure removed any probes with one or more mismatches with the target sequence. Autoradiography was carried out for 10 min

to 1 h at room temperature, using Kodak XAR5 film (EASTMAN KODAK COMPANY).

RFLP analyses

RFLP analyses were made of various DNA markers, using Southern blots. Genomic DNAs were digested with restriction endonucleases according to the procedure recommended by the manufacturer, subjected to agarose gel electrophoresis and transferred to nylon membrane (Gene Screen Plus; NEN) by the method of Southern⁷. The filters were pre-hybridized in a solution containing 50% formamide, 5× SSPE (1× SSPE; 0.15 M NaCl, 10 mM NaH₂ PO₄ 2H₂O, 1 mM EDTA), 5× Denhardt's solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll and 0.1% bovine serum albumin), 1% SDS and 100 µg/ml of denatured salmon sperm DNA at 42 °C for 16 h. Hybridization was carried out at 42 °C for 44 h in the same solution except that in addition the probe radiolabelled by the random primer method with ³²P-dCTP was included. After hybridization, the filters were washed twice with 2× SSPE at room temperature for 10 min, with 2× SSPE/0.5% SDS at 65 °C for 20 min and 0.5× SSPE at 65 °C for 10 min. The filters were exposed to Kodak XAR-5 film at -70 °C for 1-5 days with an intensifying screen (Dupont).

Detection of circulating anti-heart antibodies (AHAbs)

Circulating AHABs in 59 patients with DCM were detected using an indirect immunofluorescence technique⁸ and sections of normal human heart obtained from autopsy specimens.

Statistical analysis

The strength of the statistical association between DCM and genetic markers was expressed by the relative risk (RR) as given by Woolf⁹ and the statistical significance was examined by chi-square test with Yate's correction. A p value of less than 0.05 was considered to have statistical significance.

RESULTS

Circulating AHABs

The rate of occurrence of circulating

TABLE V ASSOCIATION BETWEEN DCM AND IGLV

Genotype	Control n=127	DCM			
		Total n=61	<45yo n=33	Ab (+) n=35	Ab (-) n=24
1/1	28.3%	14.8%	15.1%	17.2%	12.5%
1/2	52.8	47.5	39.4	51.4	45.8
2/2	18.9	37.7*	45.5**	31.4	41.7***

Ab: anti-myocardial antibody, Enzyme: *Bam*HI allele1: 5.3 kb, allele2: 3.3 kb, Probe: Immunoglobulin λ light chain gene IGLV (pV 3.3)

*RR=2.6 $p<0.01$, **RR=3.6 $p<0.005$, ***RR=3.1 $p<0.01$

TABLE VI ASSOCIATION BETWEEN DCM AND TCRA

Genotype	Control n=45	DCM			
		Total n=61	<45yo n=33	Ab (+) n=35	Ab (-) n=24
1/1	8.9%	22.0%	22.6%	31.4%*	4.2%
1/2	46.7	35.6	25.8	45.7	25.0
2/2	44.4	42.4	51.6	22.9	70.8**

Ab: anti-myocardial antibody, Enzyme: *Taq* I allele1: 8.0 kb, allele2: 2.1 kb, Probe: T cell receptor α gene TCRA (pY 14)

*RR=4.7 $p<0.01$, **RR=3.0 $p<0.05$

AHAb positive sera was 59% in patients with DCM. In this study, 22 patients were analysed at the time of their first diagnosis, and 19 of them (86%) had circulating AHAb.

Frequencies of HLA class II alleles.

As shown in Table III, the frequencies of HLA-DQB1*0503 (15% vs 5%; RR=3.06, $\chi^2=7.19$) and DQB1*06604 (21% vs 10%; RR=2.41, $\chi^2=6.20$) were significantly increased and that of HLA-DQB1*0502 (RR=1.74) was slightly increased in DCM patients. The frequency of DQB1*0303 (16% vs 31%; RR=0.44, $\chi^2=5.16$) was significantly decreased as was the frequency of HLA-DQA1*0301 (68% vs 54%; RR=0.55, $\chi^2=4.55$) (Table IV).

RFLP analyses

Among the genes tested, IGLV was found to be associated with the susceptibility for DCM. A 3.3 kb *Bam*HI subfragment from lambda V4A¹⁰ was used as probe for IGLV and two alleles represented by polymorphic *Bam*HI fragments of 5.3 kb (A1) and 3.3 kb

(A2) were detected. Distribution of these alleles seemed to follow Hardy-Weinberg's law and the allele frequencies for A1 and A2 were 38.5% and 61.5%, respectively, in patients with DCM, compared with the 54.7% and 45.3%, respectively in the healthy controls. The A2/A2 genotype was observed in only 18.9% of Japanese control subjects (24 of 127), whereas the frequency of this genotype was 37.3% in DCM (RR=2.6, $\chi^2=7.8$), 45.5% in the patients under age 45 years (age at diagnosis) (RR=3.6, $\chi^2=10.0$) (Table V).

The DNA probe TCRA (T cell receptor α gene)¹¹ recognizes *Taq*I endonuclease polymorphism identified by two alleles, a 8.0 kb fragment (A1) or a 2.1 kb fragment (A2) in addition to 2.4 kb constant fragments. No significant differences in allele frequencies were present between DCM patients and controls. However, when DCM patients were classified into two groups according to the presence of circulating AHAb, TCRA polymorphism was found to be significantly associated with circulating AHAb (Table VI).

DISCUSSION

Although dilated cardiomyopathy by definition is a condition of unknown cause, it is likely to be a syndrome stemming from a wide variety of etiologic insults to the myocardium. The possible causes or conditioning factors that contribute to the development of DCM include genetic predisposition, viral myocarditis associated with immunological abnormalities, alcohol or other toxic substances, pregnancy, or endocrine disorders. While there is little evidence of a familial tendency for DCM, there are reports describing familial DCM¹²⁻¹⁵. In studies of the natural history of DCM, 6% to 20% of patients were found to have a family history of the disease. Although the mode of inheritance is often regarded as uncertain, several authors have suggested an autosomal recessive or X-linked related cause. Yamaguchi and Toshima¹⁶ considered that the mode of inheritance is autosomal recessive because of the high incidence of consanguineous marriage among grandparents of patients with DCM. Berko and Swift¹⁷ reported a large kindred in which X-linked inheritance of DCM was suggested, onset in males was early and in females was late; there was no evidence of male to male transmission.

Immunological dysfunctions such as the presence of auto-antibodies to the myocardium¹, persistent infection of Coxsackie B virus² and deficient natural killer cell activity¹⁸ have been noted in the patients with DCM. A relationship with prior acute myocarditis has long been suspected. The hypothesis that DCM often results from previous recognized or unrecognized viral myocarditis has also been discussed¹⁹. The evidence supporting this hypothesis includes documentation of progression from acute viral myocarditis to chronic DCM, the high incidence of enteroviral antibodies²⁰ in the sera of DCM patients and identification of persistent inflammation in the heart of patients with DCM. Quigley et al²¹ found that out of 23 patients who presented with clinical and histological evidence of acute myocarditis, 12 (52%) developed dilated cardiomyopathy during an average follow-up period of 43 months. Immune responses to foreign antigens, e.g. viral antigens, are

known to be controlled by genetic factors, such as genes for HLA antigens, immunoglobulins and T cell receptors.

Among the genetic factors, HLA is an immune-control gene and is well analysed. Availability of the PCR technique facilitates a precise identification of allelism of HLA class II genes, i.e. HLA-DNA typing²²⁻²⁵. Some studies suggested an association between DCM and specific HLA antigen e.g. DR4, DR4/DQw4, DR5 or B27 (positive association) and DR3 or DR6 (negative association)²⁶⁻³¹. Limas and Limas³⁰ described that 67% of the anti-beta-receptor antibody positive patients with DCM were typed as HLA-DR4, compared to only 10% of the antibody negative patients. In our study, we found strong association between specific DQB1 alleles and DCM. Our results suggest the existence of a gene in linkage disequilibrium with the DQB1 gene, thus the virus-immune hypothesis of DCM is given support. Based on known sequences, DQB1 alleles can be classified into three subgroups by the thirtieth amino acid residue histidine, serine and tyrosine (Table II). It is of interest that frequency of the DQB1 alleles in the histidine group were increased in DCM. Furthermore, the DQB1-0604 allele was strongly associated with Auto-AHAb positive patients with DCM. Thus, the DQB1 molecule may play a role in the pathogenesis of DCM. This is apparently the first report of an association between specific DQB alleles with DCM in Japanese patients.

As described above, a correlation between DCM and the HLA system was noted in previous reports, but there is little documentation of other genetic markers. As dilated cardiomyopathy is thought to be a multifactorial disease, we also tested the correlation between DCM and various DNA markers. IGLV was seen to be strongly associated with DCM, thereby indicating that a gene in close linkage disequilibrium with the IGLV-A2 allele, especially in the homozygous state, is involved in the pathogenesis of DCM, presumably by modifying immunological responses. It is generally considered that genetic factors are more strongly associated with expression of the disease in younger patients with a multifactorial disease such as DCM, while elderly patients may develop the disease in relation

to environmental factors. This may explain why the association of DCM with IGLV was observed predominantly in the younger patients.

The T cell receptor is an important molecule for antigen recognition, in the context of HLA molecules. In the present study, TCRA polymorphism was associated with the existence of circulating AHABs. The circulating AHABs positive patients have the A1/A1 genotype (31.4%) and negative patients have the A2/A2 genotype (70.8%), predominantly. It should be noted that a large number of circulating AHABs positive patients were identified at the time of the first diagnosis. If the titer of circulating AHABs decreased gradually, some factor which destroys heart muscle, e.g. viral infection, may trigger the development of DCM. However, if the titer of AHABs does not change in the clinical course, AHABs may be related to the prognosis of DCM. A follow-up study of the patients analysed here will clarify these ideas.

Our observations suggest that genetic factors play a role in the pathogenesis, or are incremental in DCM, through diverse immune responses. Because DCM is a multifactorial disease, evidence of a genetic predisposition will aid in elucidating mechanisms involved in the development of this disorder. Detection of causative foreign antigens from viruses can now be done by subgrouping the DCM patients according to genetic background.

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