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ASSOCIATION OF HLA-DR ANTIGENS WITH DISEASE SEVERITY IN JAPANESE PATIENTS WITH RHEUMATOID ARTHRITIS

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INDEXING WORDS

rheumatoid arthritis; HLA-DR; HLA-DR4; restriction fragment length polymorphism (RFLP)

SYNOPSIS

Twenty eight Japanese patients with rheumatoid arthritis (RA) were tested for HLA-DR antigens to investigate genetic influence on disease severity and prognosis. The severity of RA was assessed by clinical, laboratory and radiological indexes. HLA-A, B, C and DR typing was performed using sera described at the 3rd Asia and Oceania Histocompatibility Workshop. HLA-DR4.1 was significantly higher in RA patients (50%) compared with controls (27.6%). HLA-DRw53 was also higher in RA patients, although DRw52 was lower in those than controls. When the relationship between HLA-DR antigens and the clinical severity of RA was investigated, the patients with HLA-DR4 had a severer disease than DR4 negative patients. In contrast, HLA-DR2 positive patients had significantly better functional scores than DR 2 negative patients. We next investigated specific genes likely to be directly involved in the pathogenesis of RA. Although the new restriction fragment length polymorphisms (RFLPs) were found in DR2 positive patients, no association was found between the RFLPs and the clinical severity

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of RA. Furthermore there were no differences in the frequencies of the RFLPs in HLA-DR4 positive patients compared with DR4 negative patients. It may be concluded that the severity of RA appears to be influenced by several different genes or molecules rather than by a single gene or gene product linked to HLA-DR2 or DR4.

INTRODUCTION

Both genetic and environmental factors have an important role in the etiology of rheumatoid arthritis (RA). An association of HLA-Dw4,[23] DR4[14,18,24] or DRw53[16,21] with RA has been observed indicating immunogenetic influences on the pathogenesis of this disease. Studies have usually been performed on populations ascertained from hospital records. Such patients are likely to be the severer RA sufferers. A recent population-based study failed to show an association with HLA-DR4 in RA patients, except with those having severer, erosive, seropositive disease.[3] Furthermore, some authors reported an association between HLA-DR4 positive RA patients and severe clinical course,[17,19,27] seropositivity[5] or high titers of rheumatoid factor.[15] It is possible therefore that HLA-DR4 or a gene linked to it might determine only the severity of an existing disease but not the primary predisposition. The observation that clinically milder disease occurs in HLA-DR2 positive RA patients when compared with DR4 positive RA patients supports the hypothesis that DR antigens may influence disease severity.[8] It was recently reported that the frequency of a new restriction fragment length polymorphism (RFLP) was increased in RA patients compared with controls and that it could be a new common susceptibility marker for RA.[7,22] Therefore, we investigated the HLA-DR antigens and RFLP of genomic DNAs in RA patients and the correlation of these data with the clinical and radiological parameters of the disease.

MATERIALS AND METHODS

Patients

Twenty-eight patients with definite or classical RA as defined by American Rheumatism Association (ARA) criteria were studied. All were Japanese, aged from 28 to 76 years old (mean:

54.5), 24 females and 4 males. They have been followed for 1 to 23 years (mean: 8.5) at the Department of Orthopedic Surgery, Kobe University Hospital. All patients were tested on several occasions for IgM rheumatoid factor (RF). Twenty-five patients were seropositive, while 3 patients had negative results when repeatedly tested for RF. Twenty-one patients were treated with sodium aurothiomalate and/or D-penicillamine and 7 with systemic corticosteroids.

Clinical assessment

The severity of the RA was assessed by clinical, laboratory and radiological indexes. Clinical assessment included patients' estimates of the duration of morning stiffness, a joint count of the number of swollen and tender joints, and the Westerngren erythrocyte sedimentation rate (ESR) according to the modified articular index of Lansbury[12] (maximum possible = 131). Functional capacity was determined by scoring activities of daily living according to the modified Lievre criteria,[13] which consists of eleven graduations (maximum possible = 100). Hand and wrist X-ray films were examined as anatomic indicators of disease severity and graded according to the criteria of Steinbrocker (1 = normal or periarticular osteoporosis, 2 = a few small cortical erosions, 3 = large multiple erosion or joint deformities, 4 = bony ankylosis).[26]

HLA typing

Mononuclear cells were separated from heparinized blood by Ficoll-Hypaque gradient centrifugation. After agglutination of platelets and monocytes with thrombin-enriched lymphocytes, they were incubated in a plastic straw column containing nylon wool. Non adherent cells were collected by dripping medium through the column, and adherent cells were recovered by squeezing the straw. Non adherent cells were used for HLA-A, B, and C typing. The lymphocyte cytotoxic test was performed using the 3rd Asia Oceania Histocompatibility Workshop method. The antisera used for determination of the DR alleles were those designated by the 3rd Asia and Oceania Hisotcompatibility Workshop and DR4 was classified into DR4.1 and 4.2.

Analysis of genomic DNA

Genomic DNA was extracted from peripheral blood leukocytes of RA patients.[11] DNA (5-15 μg/ml) was digested for 16 hr with EcoRI or Pst I (Takara, Kyoto, Japan) at 37C. The resulting DNA fragments were separated on 0.7% agarose gels by electophoresis at 30 V for 18-20 hr and were transferred to nitrocellulose filters (Paul, Gren Coove, NY) by the method of Southern.[25] Human HLA class II cDNA probes (DQα(pDCa107) and DQβ(pDCβ101))[10] were labelled with α-[^{32}P]-dCTP (Amersham) by nick-translation. Before hybridization, the nitrocellulose filters were baked for 2 hr at 80C and were prehybridized for 6-12 hr at 65C in 5 x SSPE (1 x SSPE = 0.18 M NaCl/10 mM sodium phosphate/1 mM EDTA)/0.2% sodium dodecyl sulfate (SDS)/5 x Denhardt's reagent and 100 g/ml denatured salmon sperm DNA. The filters were then hybridized with the ^{32}P-labelled heat-denatured probe for 18-20 hr at 65C. The filters were washed three times at about 50C for 30 min each with PBS (pH 7.0) / 1 mM EDTA / 0.2% SDS. The labelled fragments were detected by autoradiography using Fuji RENOX film with intensifier at -80C for 2-3 days.

Statistical Analysis

Data were analysed by the chi-square and Student's t-test distribution. Relative risk (RR) was determined by the formula RR = (P+)x(C-)/(P-)x(C+), where P and C denote the numbers of positive and negative for the specificity, respectively.

RESULTS

Distribution of HLA antigens in RA patients as compared with controls

There were no differences in the frequencies of HLA-A, B, and C antigens between the patients and control population (data not shown). Table 1 shows the frequencies of the relevant HLA-DR antigens in the two groups. HLA-DR4 was somewhat higher in RA patients, with a frequency of 57.1% as compared to 38.8% for controls. When HLA-DR4 was classified into DR4.1 and DR4.2, a significant increase in DR4.1 was observed in RA patients. HLA-DR 4.1 was present in 50.0% of RA patients and in 27.6% of controls ($p < 0.05$, not corrected). The relative risk was 2.63. An increase

Table 1 Frequencies of HLA-DR antigens in RA patients and controls.

HLA-DR antigen	RA (N=28)		control (N=98)		x	relative risk	p value
	N	%	N	%			
DR 1	4	14.3	12	12.2	-	-	NS *
DR 2	9	32.1	33	33.7	0.02	0.93	NS
DR 4	16	57.1	38	38.8	3.68	2.11	NS
[DR 4.1	14	50.0	27	27.6	5.00	2.63	p<0.05
[DR 4.2	2	7.1	11	11.2	0.39	0.61	NS
DR 5	3	10.7	18	18.4	-	-	NS
DR 6	1	3.6	19	19.4	-	-	NS
DR 7	0	0.0	1	1.0	-	-	NS
DRw8	6	21.4	31	31.6	-	-	NS
DRw9	11	39.3	22	22.4	3.19	2.24	NS
DRw52	8	28.6	56	57.1	7.11	3.33	p<0.01
DRw53	24	85.7	58	59.2	5.63	4.14	p<0.02

p values are for comparisons of differences between RA patients and controls according to the Student's t test.
* Not significant

in the frequency distribution of DRw9 and DRw53 was also observed. HLA-DRw53 was significantly higher in RA patients, although DRw52 was lower in those than controls. There were no differences between RA patients and controls in the frequency distribution of the other HLA-DR antigens.

The relationship between HLA-DR antigens and the clinical severity in RA patients (Table 2)

Because of possible influences of different immune phenomena on the clinical course of RA, we investigated the relationship between HLA-DR antigens as immunogenic factors and the clinical severity of RA patients. No differences were found in mean age at onset and disease duration: examination of the Lansbury's

Table 2 The relationship between HLA-DR antigens (DR2, DR4, DRw9) and clinical severity in RA

HLA-DR antigen	Number of patients	Duration of disease	Age of onset (year)	Functional score	Lunsbury index	Radiologic erosions (% grade III or IV)
DR2 positive	7	5.6±6.2	53.0±15.5	28.6±16.8*1	37.1±32.9	28.6
DR2 negative	8	10.1±4.5	46.6±9.0	52.5±24.9	47.0±21.4	75.0
DR4 positive	11	9.9±5.2	47.4±12.1	49.1±23.4*2	50.5±26.7*3	72.7*4
DR4 negative	4	2.8±2.9	55.8±12.9	20.0 ± 8.2	20.0 ± 8.6	0
DRw9 positive	7	8.3±5.5	47.7±10.2	42.9±26.3	42.4±23.2	57.1
DRw9 negative	8	7.8±6.1	51.3±14.5	40.0±23.9	42.4±31.3	50.0

Results are expressed as mean values ± standard deviation. Correlation between clinical parameters (duration of disease, age of onset and functional score), Lunsbury index, radiological changes and HLA-DR antigens.*1, *2, *3 : p<0.05, *4 : p<0.03

articular index and X-ray grade showed a trend towards HLA-DR2 positive patients having a milder disease. Seven patients with DR2 had significantly ($p < 0.05$) better functional scores than DR2 negative patients. In contrast, HLA-DR4 positive patients had significantly worse functional scores and higher articular Lansbury indexes and developed severer radiological alterations. There were no differences in the clinical severity between DRw9 positive and DRw9 negative patients.

Southern blot hybridization analysis of HLA class II locus

New HLA DNA polymorphism associated with RA was recently reported.[7,22] We found that the clinical severity of RA was positively associated with HLA-DR4 and negatively associated with DR2. Therefore we next investigated specific genes likely to be directly involved in the pathogenesis of RA. Figure 1 and 2 show Southern blot patterns obtained using the DQα probe and Pst I or Eco RI to digest DNA. Most of HLA-DR2 positive RA patients have DQ

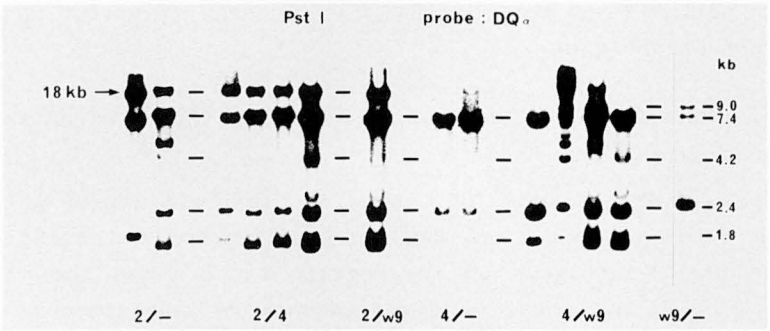

Figure 1 Southern blot of DNA from DR2, Dr4, DRw9 positive patients with rheumatoid arthritis, digested with Pst I and hybridized with the DQα cDNA probe.

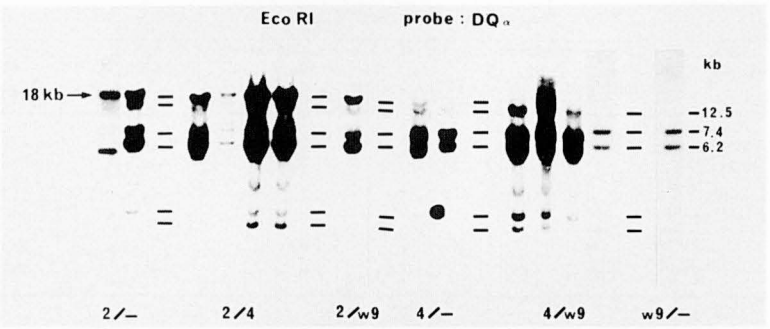

Figure 2 Southern blot of DNA from DR2, DR4, DRw9 positive patients with rheumatoid arthritis, digested with Eco RI and hybridized with the DQα cDNA probe.

α Pst I 18.0 kb (100%), DQ α EcoRI 18.0 kb (100%) and DQ β Pst I 12.0 kb (86%) fragments, which were detected in DR2 negative patients at lower frequencies (25%, 29% and 25%, respectively) (Table 3). In contrast, there were no differences in the frequencies of the RFLPs in DR4 positive patients as compared with DR4 negative patients. The frequency of DQ α Pst I 9.0 kb was significantly higher, whereas that of DQ α Eco RI 18.0 kb was significantly lower in DRw9 positive patients than DRw9 negative patients.

The relationship between the RFLPs and the clinical severity of RA was also investigated. Although HLA-DR2 positive patients had a milder disease and showed higher frequencies of DQ α Pst I 18.0 kb and EcoRI 18.0 kb fragments, no associations were observed between the RFLPs and the clinical severity of RA.

Table 3 The relationship between HLA-DR antigens and RFLPs

HLA-DR antigens	DQ_α PstI 18.0 kb		DQ_α PstI 9.0 kb		DQ_α EcoRI 18.0 kb		DQ_β PstI 12.0 kb	
	%		%		%		%	
DR2 positive	100	p<0.01	0	p<0.05	100	p<0.03	85.7	p<0.05
DR2 negative	50.0		38.9		56.3		2.5	
DR4 positive	53.6	NS*	12.5	NS	69.2	NS	43.8	NS
DR4 negative	81.8		45.5		75.0		63.6	
DRw9 positive	54.5	NS	45.5	p<0.05	44.4	p<0.05	54.5	NS
DRw9 negative	75.0		6.3		87.5		50.0	

Correlation between HLA-DR antigens and restriction fragment length polymorphisms (RFLPs)
* Not significant

DISCUSSION

Rheumatoid arthritis is the first disease that has been specifically associated with a D locus determinant (DR4) and not with an HLA-B locus antigen.[18,24] Similar results were observed in Japanese patients with seropositive RA.[14] A recent study failed to show an association of HLA-DR4 with RA patients except with those having a severer disease.[3] Our results did not show a significant positive association with DR4 in RA patients. The failure of the RA group to show this positive association may be due to the small number of patients compared with the control

group. However, a strong association was observed between HLA-DR4.1 and RA in our study. It is reported that three HLA-DR4 serological patterns (DR4.1, 4.2, 4.3) can be recognized, which closely correspond to the Dw associations with DR4. HLA-DR 4.1 is the most common in Caucasoids, being found in approximately 70% of the DR4 positive population. Most Caucasian patients with RA have the major pattern (DR4.1). This means that HLA-DR4.1 or the genes linked to it may act as a susceptibility gene for RA. In addition, a strong association between HLA-DRw53 and RA was observed. Sasazuki also reported this association in Japanese patients with RA. The association of HLA antigens with the disease has generally been thought to be due to the linkage desequilibrium between the HLA genes and the genes which predispose to the diseases. Thus, these findings indicate that both HLA-DR4.1 and DRw53 may be important in conferring disease in Japanese patients with RA and that D-DR region may be important in coding for possible Ir genes.

Since RA is a disease with many clinical and serological course differences, we investigated whether there might be disease patterns correlating to different HLA-DR antigens. This question was stimulated by some reports of an association between HLA-DR4 positive RA patients and severe clinical course,[17,19,27] seropositivity[5] or high titers of rheumatoid factor.[15] Furthermore, a strong association has been shown with extraarticular disease such as Felty's syndrome[4] and the RA patients homozygous for DR4 may suffer from severer disease than those heterozygous.[1] From our data, we can support the suggestion that HLA-DR4 positive patients are a distinct subgroup of RA. HLA-DR4 positive patients had worse functional scores, higher articular Lansbury indexes and developed severer radiological alterations. Thus, HLA-DR4 or a gene linked to it may act as either a susceptibility or a severity gene for RA. In addition to this positive association of clinical severity of RA with HLA-DR4, we have also identified a negative correlation of severity with the presence of DR2. A very mild course of RA was observed in the HLA-DR2 positive group: these patients had better functional scores and a slower progress of radiological changes. HLA-DR2 has been shown to be a marker of resistance against RA[8] as well as against type I diabetes mellitus[9] or penphingus vulgalis,[20] but it is not supported by our results. Therefore, it may be concluded that HLA-DR2 has some protective effect on the course of RA. It is possible that HLA-DR4

and DR2 antigens or genes linked to them may influence clinical severity of RA.

The HLA class II region is considered to consist of five α-chain genes and eight β-chain genes; there are encoded in three subregions, HLA-DP, -DQ and -DR. Certain alleles of HLA-DR series are in strong linkage disequilibrium with alleles of the DQ series. Lately, specific genotypic markers, such as the DQα or DQβ cDNA probe, have been used for the molecular characterization of phenotypically similar haplotypes associated with distinct diseases. A recent report indicates that the frequencies of DQβ Taq I 6 kb RFLP[7] or DQw3.1 allele, identified by 3.4 kb (Hind III), 2.3 kb (Sst I), and 3.7 kb and 6.9 kb (Bam HI) RFLPs[22] are higher in HLA-DR4 positive RA patients compared with DR4 positive controls, and that these could be new common susceptibility markers in RA. We next investigated specific genes likely to be directly involved in the pathogenesis of RA. HLA-DR2 positive patients showed an increase in the frequencies of DQα Pst I 18.0 kb, DQα EcoRI 18.0 kb and DQβ Pst I 12.0 kb fragments. Since no associations were observed between the appearance of the fragments and the clinical severity in RA, it is not certain whether genes represented by these RFLPs might have some protective effect on the course of RA. On the other hand, there were no differences in the frequencies of the RFLP in HLA-DR4 positive patients as compared with DR4 negative patients, whereas a specific DQα or DQβ variant associated with DR4-positive RA patients was reported.[7,22] This difference may be due to heterogeneity between the populations studied. These results suggest that the prognosis of RA might not be influenced by a single gene, but that some genes linked to HLA class II region might govern severity of this disease. The observation that properdin factor phenotypes are associated with RA and that this association can be independent of linkage disequilibrium with HLA-DR4[6] suggests that there are genes in the major histocompatibility complex (MHC) which are separate from the DR locus and govern susceptibility or severity. Thus it is possible that a disease susceptibility gene could be associated with the full spectrum of the disease, whereas a disease severity gene may only be associated with the more or less severe forms. From these facts it could be concluded that there is not a single gene and/or gene product of HLA-DR4 or DR2 but several different genes or

molecules, possessing complementary functions with hierarchical as well as additive effect, may influence disease severity of RA.

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