HLA-DR4 and Gm(1,3;5,21) are associated with U1-nRNP antibody positive connective tissue disease

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SUMMARY Patients with U1-nRNP antibodies (n=35, 31 female, four male) were typed for HLA-A, -B, -C, and -DR antigens and IgG heavy chain allotypes G1m(1), -(2), -(3), G3m(5), and -(21). The patient group was clinically heterogeneous. Four met the American Rheumatism Association criteria for systemic lupus erythematosus, six for progressive scleroderma, and 14 for rheumatoid arthritis. Sicca syndrome was present in seven cases. Twenty three had overlapping features compatible with mixed connective tissue disease (MCTD). Healthy blood donors served as controls for HLA typing (n=64), Gm typing (n=228), or both (n=56). Sixty six per cent of the patients with U1-nRNP antibodies were DR4 positive compared with 28% of the controls (relative risk=4.9, p=0.00053). The Gm(1,3;5,21) phenotype was found in 46% of the patients and 25% of the controls (relative risk=2.47, p=0.0247). Within the patient group Gm(1,3;5,21) was found only in DR4 positive individuals. The coincidence of HLA-DR4 and Gm(1,3;5,21) increases the relative risk values to 8.0 (compared with the group with neither risk factor). DR4 and Gm(1,3;5,21) primarily seem to be related to U1-nRNP antibody formation and not to disease expression. Patients with or without MCTD did not differ with respect to DR4 or Gm(1,3;5,21) frequency. Disease onset was earlier in patients with HLA-DR4/Gm(1,3;5,21) than in patients without both markers (mean 27.9 v 40.1 years; p<0.05).

Key words: immunoglobulin allotypes, major histocompatibility complex, mixed connective tissue disease.

Antibodies to nuclear U1-ribonucleoprotein (U1-nRNP) have been described as a serological marker for patients with overlapping features of systemic lupus erythematosus (SLE), progressive scleroderma (PSS), polymyositis (PM),^{1,2} and rheumatoid arthritis (RA).³ Patients with U1-nRNP antibodies most frequently have Raynaud's phenomenon, arthritis, swollen hands, and other scleroderma-like cutaneous or visceral features and myositis.¹⁻⁶ Forty five to 74% of patients with U1-nRNP antibodies in high titres can be classified as mixed connective tissue disease (MCTD).^{1,2,4-6} The nosological entity of MCTD has been much debated because clinical picture, disease course, and prognosis are highly

variable.^{7,8} Moreover, many patients can be classified as PSS⁷ or SLE with a low incidence of double stranded DNA antibodies or nephritis.⁹

During the last years much evidence has been obtained that autoantibody formation is influenced by genetic factors. HLA-DR3 has been found to be associated with antibodies to cell nuclei¹⁰ and collagen type II¹¹ in RA, with antibodies to SS-A (Ro) and SS-B (La) in SLE¹² and primary Sjögren's syndrome,^{13,14} especially in high titres,¹⁵ with antibodies to double stranded DNA¹⁶ and high titres of antibodies to single stranded DNA¹⁷ in SLE, with Jo-1 antibody in polymyositis,¹⁸ and with increased platelet associated IgM in chronic idiopathic thrombocytopenia.¹⁹ Moreover, HLA-DR2 is increased in SS-B antibody positive SLE patients,¹³ in lupus patients with antibodies to double stranded DNA,²⁰ and with antibodies to type II collagen in RA.²¹ In patients with scleroderma an association of

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HLA-DR1 and antibodies to the centromere region has been reported.²²

In addition, there is evidence from studies in man and animals that alleles of immunoglobulin α and γ chains are genetically linked to the humoral immune response to certain xeno-²³⁻²⁶ and autoantigens.²⁷⁻²⁹

So far, the search for HLA associations in patients with U1-nRNP antibodies has not been successful.^{17 30 31} We report here an increased frequency of

the HLA antigens DR4 in U1-nRNP antibody positive patients with chronic inflammatory rheumatic disease, and an interactive effect of the Gm(1,3;5,21) phenotype with HLA-DR4.

Subjects and methods

PATIENTS

We studied 35 patients (four male, 31 female)

Table 1 Clinical, laboratory, and immunogenetic data for 35 patients with U1-nRNP antibodies

Patient No	Sex	Age at onset (years)	Raynaud's syndrome	Puffy fingers	Sclerodactyly	Finger tip necrosis	Other PSS features	Rash/photosensitivity	Myositis	Arthralgia	Swollen joints	Pulmonary involvement	Oesophageal dysmotility	Peripheral nerve system	Leuco-/thrombocytopenia	Sicca syndrome	Lymph node/spleen	Hypergammaglobulinaemia
1	F	18	+							+	+			PN			N	
2	F	10	+	+						+	+							
3	M	21	+		+	+			+	+	+		+		L		N	+
4	F	18	+	+						+	+							+
5	M	43	+	+	+					+	+							+
6	F	57	+		+		+			+	+	+						+
7	F	27	+							+	+				L			+
8	F	17	+							+	+						N	+
9	F	47	+				+			+	+			PN	L	+	S	+
10	M	34	+	+	+	+	+			+	+	+						+
11	F	26	+	+	+				+	+	+							+
12	M	35	+	+						+	+							+
13	F	42	+		+				+	+	+					+	S,N	+
14	F	18	+							+	+	+						+
15	F	26	+	+					+	+	+							+
16	F	26	+		+		+	+		+	+				L			+
17	F	25		+	+		+		+	+	+				L,T	+	S	+
18	F	37	+	+	+		+	+	+	+	+	+				+	S,N	+
19	F	43	+	+	+		+		+	+		+	+			+	S	+
20	F	13	+		+				+	+		+	+				S	+
21	F	7	+		+	+	+					+	+			+		+
22	F	32	+							+				CTS				+
23	F	25	+	+	+					+					T			+
24	F	46	+	+	+			+		+		+			L			+
25	F	33	+							+								+
26	F	25	+							+					L			+
27	F	17	+							+								+
28	F	53	+							+			+		L			+
29	F	32								+					L			+
30	F	53								+	+			CTS				+
31	F	56								+	+				L			+
32	F	52								+					L,T			+
33	F	37								+	+			PN		+		+
34	F	57	+							+								+
35	F	44	+					+		+	+			CTS	L			+
Total	M=4 F=31		29	10	14	3	8	4	7	34	22	6	6	6	13	7	N=5 S=6	27

*Joint erosions.
PN=polynuropathy; CTS=carpal tunnel syndrome; L=leucocytopenia; T=thrombocytopenia; N=lymph node enlargement; S=splenomegaly.

selected only for the presence of U1-nRNP antibodies as determined by double immunodiffusion in agarose gel and by a competitive radioimmunoassay (see below). Mean age at disease onset was 35 years (range 7–57 years) and mean disease duration 6.3 years (range 0.3–27 years). The clinical and selected laboratory data are given in Table 1. The patient group was clinically highly heterogeneous. According to established criteria four were classified as

systemic lupus erythematosus,³² six as progressive systemic sclerosis,³³ 12 as definite and two as classical (erosive) rheumatoid arthritis,³⁴ seven as Sjögren's syndrome,³⁵ and one as polymyositis.³⁶ Several patients met two or more clinical classifications. Twenty three patients fulfilled the criteria of Sharp *et al*,¹ modified by Ramos Niembro *et al*,³ for MCTD.

Other antibodies	Classification	HLA antigens				Gm phenotype
		A	B	Cw	DR	
	MCTD	3,28	35,60	3,-	4,5	1,3;5,21
	MCTD, RA	2,-	7,44	5,-	4,-	1,3;5,21
	MCTD, PSS, RA	2,-	5,62	3,-	4,-	3;5
	MCTD, RA	3,-	7,13	-,-	4,5	1,3;5,21
	MCTD, RA	2,11	27,50	2,6	1,3	1,2,3;5,21
	MCTD, RA	2,-	7,18	-,-	2,7	1,2,3;5,21
	MCTD, RA	1,3	8,39	-,-	4,6	3;5
	MCTD, RA	2,25	18,62	3,-	2,4	1,3;5,21
	MCTD, PSS	30/31,32	13,35	4,-	1,5	3;5
	MCTD, PSS, RA	2,25	44,62	4,5	4,-	1,3;5,21
	MCTD	1,2	5,8	-,-	4,-	1,2,3;5,21
	MCTD, RA	2,28	27,35	1,4	1,-	3;5
dsDNA, La	MCTD	1,3	8,35	4,-	3,4	3;5
	MCTD	1,-	35,38	4,6	7,-	1,2,3;5,21
Sm	MCTD	2,-	51,62	3,-	4,7	1,3;5,21
dsDNA, Sm, Ro	MCTD, SLE, PSS	24,30	49,60	3,-	2,4	1,3;5,21
	MCTD, SLE, PM, RA	1,3	8,18	-,-	3,4	1,3;5,21
	MCTD, SLE, RA	1,28	8,14	-,-	2,5	3;5
DNP, Ro	MCTD, PSS	2,-	7,44	3,-	2,4	1,3;5,21
	MCTD	2,-	44,62	3,-	4,-	1,3;5,21
	PSS	2,26	18,38	-,-	4,5	1,3;5,21
		1,-	7,14	-,-	4,5	1,3;5,21
		1,32	8,44	5,-	3,4	3;5
	MCTD	2,-	17,39	-,-	3,4	1,3;5,21
		2,11	22,44	3,5	3,9	3;5
		1,32	7,8	-,-	2,4	1,3;5,21
	MCTD	1,3	5,37	-,-	1,2	3;5
		3,-	7,22	-,-	2,4	1,3;5,21
		3,32	61,62	2,3	4,5	1,2;21
Sm	RA	1,24	8,60	3,-	2,4	1,3;5,21
	RA	1,2	8,27	1,2	3,5	3;5
dsDNA		2,3	5,22	1,4	2,4	1,2,3;5,21
	RA	2,-	8,13	6,-	3,7	3;5
	MCTD	3,32	7,-	2,-	2,6	3;5
Sm	SLE	2,3	7,27	2,-	2,8	1,2,3;5,21

CONTROLS

Healthy Caucasian blood donors were used as controls for typing of HLA antigens (n=64), Gm factors (n=228), or both (n=56).

DETERMINATION OF AUTOANTIBODIES

U1-nRNP antibodies and other autoantibodies (Ro, La, Sm) were determined by double diffusion³⁷ in agarose gel using antigen containing extracts from calf thymus nuclei or human spleen and reference sera kindly supplied by Dr Tan (LaJolla, USA) and from the Center of Disease Control (Atlanta, USA). In addition, sera were tested by a competitive radioimmunoassay. IgG globulins from sera monospecific for U1-nRNP antibodies were isolated by ion exchange chromatography³⁸ and coated on polyvinyl microtitre plates (10 µg/ml in carbonate buffer pH 9.5). After blocking residual protein binding capacity with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), calf thymus nuclear extract was added (700 µg/ml in saline) and incubated for one hour at room temperature. After washing (PBS containing 1% BSA and 0.05% Tween 20), patient sera (50 µl, starting dilution 1/10 in PBS) and radiolabelled³⁹ U1-nRNP IgG (50 µl; specific activity about 10 µCi/µg (0.37 MBq/µg)) were added and incubated for three hours at room temperature. The plates were washed four times, dried, cut into single wells, and counted in an automated gammacounter. The results were expressed as percentage binding of radiolabelled IgG. Serum values two standard deviations below the mean of 20 sera from healthy blood donors were regarded as inhibitory (positive). All tests were done in duplicate.

Antibodies to native DNA were determined by the *Crithidia luciliae* immunofluorescence method.⁴⁰ IgM rheumatoid factors were determined by latex agglutination tests or laser nephelometry.⁴¹

HLA TYPING

HLA antigens of the A, B, C, and DR locus were typed with the standard NIH complement dependent microcytotoxicity assay⁴² using sera purchased from and tested for specificity by Behringwerke (Marburg, FRG), Biotest (Dreieich, FRG), and Fresenius (Oberursel, FRG).

Gm TYPING

The immunoglobulin heavy chain factors G1m(1), G1m(2), G1m(3), G3m(5), and G3m(21) were tested by indirect haemagglutination with anti-D sensitised human red blood cells⁴³ on microtitre U bottom plates with reagents from Biotest (Dreieich, FRG) and Fresenius (Oberursel, FRG).

STATISTICS

The frequencies of HLA antigens and Gm phenotypes and haplotypes in U1-nRNP positive patients and controls were compared by contingency table analysis. For 2×2 tables, the null hypothesis of no association was tested by Fisher's two sided exact test. p Values were corrected for the number of comparisons made when indicated. Relative risks were estimated by calculating odds ratios according to Woolf.⁴⁴

For three way contingency tables complex interactions were investigated by fitting log-linear models according to the hierarchical principle.⁴⁵ Significance of the three factor interaction (disease risk affected by non-additive effects of two genetic markers) was assessed by examining the appropriate G² likelihood ratio statistic. As some cell frequencies were small the analysis was performed separately by adding a constant (0.5) to each cell or by using the original values. As this procedure was purely explorative (the null hypotheses were not specified a priori) the resulting p values should be considered as hints for the presence of interactions. The computations were performed by means of BMDP statistical software.⁴⁶

Student's t test was used for comparing mean values.

Results

The clinical, laboratory, and immunogenetic results are shown in detail in Table 1.

FREQUENCY OF HLA ANTIGENS

HLA-A, -B, and -C antigen frequencies in patients did not differ from those in our control group (data not shown). When comparing the HLA-DR frequencies, however, we found the DR4 frequency

Table 2 Phenotypic frequencies of HLA-DR antigens in patients with anti-U1-nRNP positive connective tissue disease (n=35) and healthy controls (n=64)

Antigen	Frequencies (%)		Odds ratio	p Values*
	Patients	Controls		
DR1	14.3	14.1	1.02	1.0 (NS)
DR2	34.3	35.9	0.93	1.0 (NS)
DR3	22.9	20.3	1.16	0.80 (NS)
DR4	65.7	28.1	4.90	0.00053
DR5	22.9	32.8	0.61	0.36 (NS)
DRw6	5.7	20.3	0.24	0.0773 (NS)
DR7	8.6	21.9	0.33	0.16 (NS)
DRw8	2.9	1.6	1.85	1.0 (NS)
DRw9	2.9	3.1	0.91	1.0 (NS)

*Fisher's two sided exact test; p values uncorrected; NS=not significant.

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Table 3 Frequencies of Gm phenotypes in patients with anti-U1-nRNP positive connective tissue disease (n=35) and in healthy controls (n=228)

	Frequencies (%)		Odds ratio	p Values*	
	Patients	Controls			
<i>Gm phenotype</i>					
Gm(1;21)	0	3.5	0	0.60	(NS)
Gm(1,2;21)	2.9	3.5	0.81	1.0	(NS)
Gm(1,2,3;5,21)	17.1	18.9	0.89	1.0	(NS)
Gm(1,3;5,21)	45.7	25.4	2.47	0.0247	
Gm(3;5)	34.3	48.7	0.55	0.15	(NS)
<i>Gm haplotype</i>					
Gm ^{1,21}	48.6	32.5	1.97	0.0847	(NS)
Gm ^{1,2,21}	20.0	22.4	0.87	0.83	(NS)
Gm ^{3,5}	97.1	93.0	2.57	0.71	(NS)
Gm hetero-zygous	65.7	47.8	2.09	0.0684	(NS)
Gm homo-zygous	34.3	52.2	0.48	0.0684	(NS)

*Fisher's two sided exact test; p values uncorrected; NS=not significant.

significantly increased in the patient group (corrected p value <0.01; Table 2).

FREQUENCY OF Gm ALLOTYPES

A comparison of Gm phenotypes, Gm haplotypes, and Gm hetero-/homozygosity in patients and controls is given in Table 3. The frequency of the Gm(1,3;5,21) phenotype was raised to 45.7% in patients compared with 25.4% in controls (uncorrected p value=0.0247), but this difference lost statistical significance after correction of p values for the number of comparisons made.

INTERACTION HLA-DR4 AND Gm FACTORS

Although the Gm(1,3;5,21) frequency was not significantly raised in the patient group as a whole, a highly significant association was found between HLA-DR4 and Gm(1,3;5,21) in U1-nRNP positive patients: 16 of 23 HLA-DR4 positive patients (69.6%) displayed the Gm(1,3;5,21) phenotype, and all 16 patients carrying Gm(1,3;5,21) were DR4 positive (p value <0.0001). In the control group no

Table 4 Combinations of DR4 and various Gm(1,2;21) phenotypic traits in anti-U1-nRNP positive connective tissue disease patients (n=35) and healthy controls (n=56)

		Number of individuals				Odds ratio*	p Values*
		Patients		Controls			
		Observed	Expected†	Observed	Expected†		
DR4	Gm(1,3;5,21) phenotype						
+	+	16	10.5	5	4.25	8.0	0.00046
+	—	7	12.5	12	12.75	1.5	0.56
—	+	0	5.5	9	9.75	0	0.0940
—	—	12	6.5	30	29.25	1	
Significance of three way interaction: with adding of constant: p=0.0036 without adding of constant: p=0.0009							
DR4	Gm ^{1,21} haplotype						
+	+	17	11.2	7	5.5	5.7	0.00199
+	—	6	11.8	10	11.5	1.4	0.75
—	+	0	5.8	11	12.5	0	0.0478
—	—	12	6.2	28	26.5	1	
Significance of three way interaction: with adding of constant: p=0.0035 without adding of constant: p=0.0009							
DR4	Gm						
+	Heterozygous	19	15.1	7	7.0	7.8	0.00052
+	Homozygous	4	7.9	10	10.0	1.2	1.0
—	Heterozygous	4	7.9	16	16.0	0.72	0.74
—	Homozygous	8	4.1	23	23.0	1	
Significance of three way interaction: with adding of constant: p=0.0276 without adding of constant: p=0.0222							

*Subjects with neither risk factor taken as reference. Fisher's two sided exact test.

†Calculated assuming independence of the two disease predisposing markers.

association of these two genetic markers could be found ($p=0.74$). In Table 4 all combinations of the presence or absence of these two disease predisposing markers are shown for our patients and controls. When individuals with neither risk factor were taken as the reference, an odds ratio estimating a relative risk of 8.0 was calculated for DR4/Gm(1,3;5,21) carriers. Similar results were obtained for the combination of DR4 and the Gm^{1,21} haplotype and for DR4 and Gm heterozygosity (Table 4). For the latter comparison we assumed individuals phenotypically typing as Gm(1,2;21) (one patient and two controls) to be heterozygotes (of Gm^{1,21} and Gm^{1,2;21}). Analysis of the data by fitting log-linear models indicated that three way interactions between DR4, Gm marker, and disease have to be assumed to describe the data (p values of the corresponding G^2 test were always <0.05 ; Table 4).

HLA-DR4 AND Gm(1,3;5,21) IN PATIENT SUBGROUPS

The markers HLA-DR4 and Gm(1,3;5,21) were similarly distributed in 23 patients classified as mixed connective tissue disease³ and in those 12 without significant overlapping (DR4: 16/23 MCTD ν 7/12 non-MCTD; Gm(1,3;5,21): 12/23 MCTD ν 4/12 non-MCTD). No association was found between HLA-DR4 or Gm(1,3;5,21), or both, and any other clinical classification or feature. The disease predisposing HLA-DR and Gm markers were associated with an early onset of disease: those 16 patients positive for DR4/Gm(1,3;5,21) had a mean age at disease onset of 27.9 (SD 14.6) years compared with 40.1 (13.5) years in those 12 patients carrying neither factor ($p<0.05$). Seventeen of the 20 patients (85%) with disease onset before the age of 35 were DR4 positive compared with 6/15 (40%) in the late onset group ($p<0.02$).

Discussion

Evidence for familial aggregation of MCTD is rare. Some single reports on families with MCTD and other connective tissue diseases in different members have been published.^{47,48} A family with two HLA identical siblings suffering from MCTD and with rheumatic symptoms and rheumatoid factors in eight of 18 relatives has been described.⁴⁹ In one of three families with U1-nRNP positive propositi studied by Bell and Maddison the inheritance was linked to the HLA-DR4 positive haplotype.³⁰

We found a significant increase of HLA-DR4 in our U1-nRNP antibody positive patients. Previous studies of U1-nRNP antibody positive patients, mainly³⁰ or exclusively^{17,31} cases with SLE, had

failed to demonstrate HLA associations with this particular subgroup. In contrast, only four cases of our group were classified as SLE, two of these were HLA-DR4/Gm(1,3;5,21) positive. Furthermore, in the study of Bell and Maddison 19 of the 64 patients were anti-Ro positive, and of these, all 10 patients tested carried HLA-DR3.³⁰ The other patients frequently had antibodies to native DNA. Both antibodies were only rarely found in our patients. These discrepancies point to different patient populations studied.

It could be argued that the high percentage of patients with DR4 in our group might be due to the fact that many of the patients also had rheumatoid arthritis, a disease with a well known DR4 association,^{10,50-52} but even in those patients without swollen joints the DR4 frequency was raised to 77% (10/13). Furthermore, only two of our patients had classical erosive rheumatoid arthritis. Likewise, 61% (11/18) of patients without serum rheumatoid factor were also DR4 positive. Finally, our U1-nRNP antibody positive patients differ from our patients with rheumatoid arthritis on a genetic basis also since the Gm(1,2;21) and not the Gm(1,3;5,21) phenotype was shown to be associated (18%) with DR4 positive seropositive rheumatoid arthritis,⁵² and only one (3%) of the U1-nRNP positive patients carried Gm(1,2;21).

In addition to the increased HLA-DR4 frequency, we found an association of the haplotype Gm(1,21) and phenotype Gm(1,3;5,21) in HLA-DR4 positive patients with U1-nRNP antibodies. The coexistence of these two independent genetic markers⁵³ from chromosome 6 and 14 markedly increased the risk of an individual acquiring or suffering from U1-nRNP antibody positive connective tissue disease. Such interactive effects have been observed in immune response to bacterial antigens,²³ in susceptibility to *Haemophilus influenzae* disease,⁵⁴ in chronic active hepatitis,⁵⁵ and in rheumatoid arthritis.⁵⁶

HLA-DR4 either by itself or in combination with the Gm phenotype Gm(1,3;5,21) does not seem to be related to disease expression. The frequency of these genetic markers does not differ between patients classified as MCTD and U1-nRNP antibody positive patients without MCTD. The same holds true for other disease classifications or single clinical features. Our results favour the hypothesis that the association of HLA-DR4 and Gm(1,3;5,21) more directly concerns the U1-nRNP antibody formation or may be more intimately related to the induction mechanism than to the clinical reaction pattern. A similar situation seems to exist for antibodies to Ro or La which are associated with HLA-DR3. This association is found irrespective of the clinical

diagnosis of SLE,¹² subacute cutaneous lupus,⁵⁷ or primary Sjögren's syndrome,⁵⁸ and in anti-Ro positive, asymptomatic mothers of babies with neonatal lupus.⁵⁸ In progressive systemic sclerosis an association with HLA-DR1 and DR5 has been described without any clinical associations,⁵⁹ but others found that the DR1 association is linked to the autoantibody formation against the centromere region.²²

HLA-DR4/Gm(1,3;5,21) was particularly increased in those U1-nRNP positive patients with an early disease onset. Such a relation between HLA markers and early disease onset has been recorded in *Myasthenia gravis*⁶⁰ and in rheumatoid arthritis.^{51 52} A possible explanation for this phenomenon might be that the genetically predisposed individuals are at a particularly high risk during a certain period of their life when hormonal or environmental factors are more likely to interact with the disease predisposing genes.

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