EXTENDED REPORT

Association of HLA-DM polymorphism with the production of antiphospholipid antibodies

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Objective: To investigate whether variation in the HLA-DM gene is important in producing a group of pathogenic autoantibodies—antiphospholipid antibodies (aPL)—on the basis that HLA class II restricted antigen presentation is involved in the production of aPL.

Methods: HLA-DMA and DMB polymorphisms were genotyped by polymerase chain reaction combined with restriction enzyme digestion in 51 white patients with primary antiphospholipid syndrome (APS), 82 with systemic lupus erythematosus (SLE) (42 with APS and 40 without APS), and 109 healthy white controls. The association with the aPL profile was examined.

Results: The distribution of DMA alleles in APS patients and in patients with APS associated with SLE was significantly different from that in controls by $4 \times 2 \chi^2$ test with 3 degrees of freedom ($p=0.035$ and 0.011 , respectively), but it was not different between SLE patients without APS and controls. The allelic distribution of DMA was also different between patients with IgG class anticardiolipin antibody or those with lupus anticoagulant (LA) and controls ($p=0.012$ and 0.007 , respectively) and between patients with and without LA among SLE patients ($p=0.035$). All these differences included the increase in DMA*0102 in the former groups.

Conclusions: The results suggest that HLA-DMA*0102 or its linked gene(s) form one of the genetic risks for the production of aPL.

It is now becoming evident that certain groups of antiphospholipid antibodies (aPL) play causative roles in thrombosis. However, the mechanisms of production of aPL are still unclear. The analyses of V genes of human monoclonal aPL suggest the involvement of antigen driven positive selection at a certain stage in generating pathogenic aPL.^{1–2} Associations between the presence of aPL or antiphospholipid syndrome (APS) and HLA class II genes have been reported, supporting this antigen driven mechanism.^{3–10}

HLA-DM are recently identified molecules that are encoded by HLA-DMA and DMB genes located between HLA-DQ and DP subregions of the human major histocompatibility complex.¹¹ They accumulate in acidic endosomal/lysosomal compartments associated with classical class II molecules, protecting the empty $\alpha\beta$ dimers of those molecules from functional inactivation by low pH. They also catalyse peptide loading by releasing class II associated invariant chain peptide or low stability ligands toward high stability $\alpha\beta$:peptide complexes.^{12–13} Thus HLA-DM molecules are involved in the antigen processing pathway of HLA class II restricted antigen presentation.

Both DMA and DMB genes show polymorphism in the third exon.^{14–15} Three polymorphic sites have been found in DMA and two in DMB. According to the combinations of these polymorphisms, four different alleles at DMA—*0101 to *0104—and five at DMB—*0101 to *0105—have been identified so far.^{14–16} The possibility arises that these polymorphisms could affect class II restricted antigen presenting pathway by altering their functions.

Knowledge about possible involvement of DM molecules in the production of aPL would be helpful for a better understanding of the mechanisms of production not only of aPL but also of other autoantibodies. In an initial response to this question, we examined the susceptibility of the HLA-DM polymorphisms to aPL production.

METHODS

Patients

We recruited 51 patients with primary APS, 42 patients with APS secondary to systemic lupus erythematosus (SLE) (secondary APS), all fulfilling the Sapporo criteria for APS¹⁷ and the American College of Rheumatology criteria for SLE¹⁸, and 40 patients with SLE but without APS (none of whom had aPL). Their median age was 43 years (range 22 to 73), and the female to male ratio was 121:12. All the patients were seen in the Lupus Unit, St Thomas' Hospital, London and were involved in this study from 1995 to 1998 after giving informed consent. The control populations were represented by 109 blood donors at Guy's and St Thomas' Hospitals, London. All patients and controls were of white race, unrelated, and born in the United Kingdom.

Genotyping of HLA-DM polymorphism

Genomic DNA was extracted from peripheral white blood cells from the patients and the healthy controls using a standard phenol-chloroform extraction procedure or by salting out methods employing a kit (Nucleon BACC; Biosciences, Coatbridge, UK) according to the manufacturer's instructions. Samples of DNA were amplified for the third exon of DMA and DMB genes by polymerase chain reaction (PCR) using primers as previously described.¹⁹ PCR cycles consisted of an initial two minute denaturation at 95°C and 30 cycles of one minute denaturation at 95°C, one minute annealing at 56°C, and one minute extension at 72°C. Amplified PCR products were then subjected to restriction fragment length polymorphism (RFLP) using Fok I, Hinf I, and Aci I endonucleases (New England Biolabs, Beverly,

Abbreviations: aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; LA, lupus anticoagulant; RFLP, restriction fragment length polymorphism

Table 1 The distribution of HLA-DM alleles in patients with antiphospholipid syndrome and controls

Allele	APS (n = 186)	PAPS (n = 102)	SAPS (n = 84)	SLE APS (n = 80)	Controls (n = 218)
DMA*0101	78.0%	79.4%	76.2%	88.8%	85.8%
*0102	17.7§	14.7	21.4¶	8.8	8.3
*0103	2.7	3.9	1.2	2.5	3.2
*0104	1.6	2.0	1.2	0.0	2.8
DMB*0101	75.8	75.5	76.2	75.0	69.7
*0102	1.1	1.0	1.2	3.8	2.3
*0103	22.0	22.5	21.4	21.3	25.7
*0104	1.1	1.0	1.2	0.0	2.3
*0105	0.0	0.0	0.0	0.0	0.0

n = number of alleles in each group.

†p = 0.035, ‡p = 0.011 v controls by 4×2 χ^2 test.§p = 0.007 (pc = 0.028), ¶p = 0.003 (pc = 0.012) v controls by 2×2 χ^2 test.

APS, antiphospholipid syndrome; PAPS, primary antiphospholipid syndrome; SAPS, secondary antiphospholipid syndrome; SLE, systemic lupus erythematosus.

Massachusetts, USA) for DMA alleles, and Hha I, Bsr I, and ApaI I (New England Biolabs) for DMB alleles. The enzymatic digestion was carried out over two hours at 65°C for Bsr I and at 37°C for the others. The digested samples were then subjected to electrophoresis in 3% or, for Bsr I, 3.5% agarose gels and visualised with ethidium bromide. At least one sample was found positive for digestion in each PCR/digestion series, showing no failure in the PCR-RFLP process. DMA and DMB alleles were determined by combination of dimorphic sites according to the reports by Carrington *et al*,^{14–15} and by Naruse *et al* for DMB*0105.¹⁶

HLA-DRB1 and DQB1 typing

In 234 individuals (127 patients and 107 controls), HLA-DRB1 and DQB1 had been genotyped by PCR with sequence specific primers (PCR-SSP). In each PCR reaction a primer pair was included that amplified human growth hormone (HGH I and II) and thus functioned as an internal positive amplification control. The primers, amplification protocol, and visualisation of the amplified products have been fully described.^{20–21}

Detection of aPL

The presence of anticardiolipin antibody (aCL) was determined by enzyme linked immunosorbent assay (ELISA) according to the standardised technique.²²

For the determination of anti- β 2-glycoprotein I antibody (a β 2GPI), ELISA was carried out as previously described^{23–24} by using gamma irradiated plates (Maxisorp; Nunc, Roskilde,

Denmark), 50 μ l of 4 μ g/ml purified human β 2-GPI (Yamasa Corporation, Choshi, Japan) in phosphate buffered saline (PBS) as an antigen, and 3% porcine skin gelatin (Sigma, St Louis, Missouri, USA) as a blocking reagent.

Antiprothrombin antibody (aPT) was tested by ELISA using gamma irradiated plates (Maxisorp), 80 μ l of 10 μ g/ml human purified prothrombin (Diagnostica Stago, Asnieres, France) in PBS as an antigen, and 0.5% gelatin as a blocking reagent, as previously described.²⁵

The tests for lupus anticoagulant (LA) were carried out according to the guidelines of the Scientific and Standardization Subcommittee on LA²⁶ using activated partial thromboplastin time and dilute Russell's viper venom time, diluted at 50% and corrected by plasma or platelets, respectively. Taipan snake venom time was used in the case of patients on warfarin.²⁷

Statistical analysis

Comparisons of allelic frequencies of DM genes between different groups of patients and controls or between each DM and DR/DQ phenotype were undertaken using χ^2 tests with Yates' correction when applicable. The probability (p) values were further corrected by multiplying the number of comparisons (pc) where needed; p (or pc) < 0.05 was regarded as statistically significant. For those two genes with the possibility of linkage disequilibrium, the delta (Δ) values—that is, the correlation coefficients in the 2×2 tables—were also calculated.

Table 2 The distribution of HLA-DM alleles and antiphospholipid antibodies

Allele	aCL		a β 2GPI		aPT		LA (n = 124)	Controls (n = 218)
	IgG (n = 134)	IgM (n = 46)	IgG (n = 90)	IgM (n = 18)	IgG (n = 38)	IgM (n = 44)		
DMA *0101	76.1%	76.1%	81.1%	83.3%	84.2%	81.8%	75.8%	85.8%
*0102	20.1§	19.6	14.4	16.7	10.5	18.2	21.0¶	8.3
*0103	2.2	2.2	2.2	0.0	5.3	0.0	1.6	3.2
*0104	1.5	2.2	2.2	0.0	0.0	0.0	1.6	2.8
DMB *0101	75.4	73.9	75.6	72.2	71.1	75.0	75.0	69.7
*0102	1.5	0.0	0.0	0.0	2.6	0.0	0.0	2.3
*0103	23.4	23.9	24.4	27.8	23.7	25.0	23.4	25.7
*0104	0.7	2.1	0.0	0.0	2.6	0.0	1.6	2.3
*0105	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

n = number of alleles in patients with positive antibodies.

†p = 0.012, ‡p = 0.007 v controls by 4×2 χ^2 test.§p = 0.002 (pc = 0.008), ¶p = 0.001 (pc = 0.004) v controls by 2×2 χ^2 test.a β 2GPI, anti- β 2GPI antibody; aCL, anticardiolipin antibody; aPT, anti-prothrombin antibody; LA, lupus anticoagulant.

Table 3 Frequencies of DMA alleles in patients with or without antiphospholipid antibodies (aPL) in SLE

Allele	aPL +/– (n = 84/80)	aCL +/– (n = 70/94)	αβ ₂ GPI +/– (n = 50/110)	aPT +/– (n = 32/120)	LA +/– (n = 58/90)
DMA*0101	76.2%/88.8%	75.7%/87.2%	78.0%/83.6%	81.3%/82.5%	75.9%/86.7%
*0102	21.4/8.8	21.4/10.6	20.0/13.6	18.8/14.2	24.1/8.9†
*0103	1.2/2.5	1.4/2.1	2.0/1.8	0.0/2.5	0.0/3.3
*0104	1.2/0.0	1.4/0.0	0.0/0.9	0.0/0.8	0.0/1.1

n = number of alleles in patients with positive aPL v patients with negative aPL among SLE patients. aCL, αβ₂GPI, and aPT contain both IgG and IgM class.

†p = 0.035 by 4×2 χ² test.

‡p = 0.021 (pc = 0.084) by 2×2 χ² test.

αβ₂GPI, anti-β₂GPI antibody; aCL, anticardiolipin antibody; aPT, anti-prothrombin antibody; LA, lupus anticoagulant.

RESULTS

The distribution of HLA-DM alleles in patients and controls

The allelic frequencies of DMA and DMB genes among controls were comparable with those previously reported on white populations,^{14–15, 28} indicating the successful genotyping of these genes (tables 1 and 2).

The distribution of DMA alleles was significantly different between APS patients or secondary APS patients and controls by 4×2 χ² testing (p = 0.035 and 0.011, respectively). Among four DMA alleles, the increase in DMA*0102 showed the strongest contribution with these differences (p = 0.007 and 0.003, pc = 0.028 and 0.012, respectively, by 2×2 χ² test). The distribution of DMA alleles in primary APS patients or SLE patients without APS was not significantly different from that in controls (table 1).

In the same manner, the significant difference of the distribution of DMA alleles was observed between patients with IgG aCL or with LA and controls (p = 0.012 and 0.007, respectively), mainly reflecting the increase in DMA*0102 in the former groups (table 2). The allelic frequency of DMA*0102 in patients with IgG aPT was close to that of controls (table 2).

The distribution of HLA-DM alleles in patients with or without aPL among SLE patients

Among the SLE patients, the skewing of the distribution of DMA alleles, including the increase in DMA*0102, in patients with LA compared with those without LA was significant by 4×2 χ² testing (p = 0.035) (table 3). None of the DMB alleles was significantly different between patients with and without aPL among SLE patients (data not shown).

Association with the known APS related HLA genes

By the genotype analysis of 107 controls, the presence of DMA*0102 was strongly associated with that of DRB1*0701 (p < 0.0001, Δ = 0.470) and DQB1*0303 (p < 0.0001, Δ = 0.609), both of which were also closely associated with each other (p < 0.0001, Δ = 0.451). They have been known to be positively associated with APS/aPL. The presence of DMA*0102 was not positively associated with that of other known APS related genes (DQB1*0301/4, DQB1*0302, DQB1*0602/11, and DRB1*1302). Among 127 APS and/or SLE patients, p values (and Δ values) for these associations were 0.002 (pc = 0.17) (Δ = 0.302) for DMA*0102 v DRB1*0701 and 0.069 (Δ = 0.190) for DMA*0102 v DQB1*0303. In the APS patient group, they were 0.003 (pc = 0.25) (Δ = 0.343) and 0.59 (Δ = 0.093), respectively. Furthermore, in this dataset the allelic frequency of DRB1*0701 and DQB1*0303 was not significantly different between APS patients and controls (11.9% v 15.9%, p > 0.05 and 7.4% v 5.1%, p > 0.05, respectively).

DISCUSSION

The association between HLA class II genes and aPL production has been reported,^{3–10} not only showing that HLA class II or a linked gene is one of the genes leading to susceptibility to aPL production, but also suggesting that the T cell recognition of peptides bound to HLA class II molecules is required for generating aPL. Through these studies, the increased frequencies of HLA-DRB1*04, DRB1*07 (0701), DRB1*1302, DR53, DQB1*0301 (DQ7), *0302, and *0303 in APS patients have been shown.

In addition to these classical class II molecules, it has recently been revealed that HLA-DM molecules play crucial roles in HLA class II restricted antigen presentation, by studies of cell lines lacking HLA-DM, which are defective in class II restricted antigen processing.^{11–13} The presence of polymorphisms in DM genes^{14–15} raised the possibility of their involvement in the development of HLA class II associated diseases, although the relation between these polymorphisms and the function of DM molecules has not yet been clarified. Several studies of the association between these HLA-DM polymorphisms and immunological diseases have been reported, including atopic dermatitis,¹⁹ insulin dependent diabetes,^{29–30} sarcoidosis,³¹ multiple sclerosis,³² juvenile dermatomyositis,³³ rheumatoid arthritis,^{34–38} and SLE.³⁹ Some of these studies showed the presence of the association, such as the increased frequency of DMA*0103 and DMB*0102 in patients with juvenile dermatomyositis³³ and the increased frequency of DMA*0103³⁴ or DMB*0101³⁸ in French patients with rheumatoid arthritis. The study on SLE, in a Japanese population, did not find any significant association of the HLA-DM polymorphisms with the development of SLE and specific manifestations, but the existence of aPL was not considered.³⁹ We examined the susceptibility of these polymorphisms to aPL production in a white British population, and observed the skewed distribution of DMA alleles including the increase of DMA*0102 in patients with aPL, which is the first report on HLA-DM and aPL/APS. The minor effect of the DMA polymorphisms on the presence of aPT, especially of IgG class, suggested the importance of another genetic predisposition in aPT production.

It is necessary to consider the influence of other genes in linkage disequilibrium with HLA-DM genes. Several associations between HLA-DM and classical class II genes in the white population are already known, including DMA*0102 and DRB1*07-DQA1*0201-DQB1*0303 haplotype, DMA*0102, and DRB1*01-DQA1*0101-DQB1*0501 haplotype.²⁸ Consistent with this, our study in a white British population showed association of DMA*0102 with both DRB1*0701 and DQB1*0303. Thus it is possible that the haplotype DMA*0102-DRB1*07-DQB1*0303 may be important in aPL production. In this study, we were not able to determine exactly which haplotype each individual had, as we did not investigate their families. However, in our series,

neither DRB1*0701 nor DQB1*0303 was significantly associated with APS (the discrepancy from our previous report⁸ may reflect the partial difference of the population of the patients and controls). The weaker association between DMA*0102 and DRB1*0701 or DQB1*0303 in APS patients than in controls was compatible with these results. They suggest that the DMA gene itself could be influential to the production of aPL.

More than half of aPL positive patients did not have DMA*0102, indicating the implication of other genetic risk factors for aPL production separate from DMA*0102 or DMA*0102 containing haplotype. Furthermore, the presence of a considerable number of individuals with DMA*0102 but without aPL suggests the requirement of a coexistence of other genetic or environmental (for example, infections) factors for aPL production.

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