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

M L Sanchez, K Katsumata, T Atsumi, F I Romero, M L Bertolaccini, A Funke, O Amengual, E Kondeatis, R W Vaughan, A Cox, G R V Hughes, M A Khamashta

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-DM 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-DM*0102 or its linked gene(s) form one of the genetic risks for the production of aPL.

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. 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 Ava I endonucleases (New England Biolabs, Beverly, Massachusetts).

Abbreviations: aPL, antiphospholipid antibodies; APS, antiphospholipid syndrome; LA, lupus anticoagulant; RFLP, restriction fragment length polymorphism.
Massachusetts, USA) for DMA alleles, and Hha I, Bsr I, and ApaL 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% agarose gels and visualised with ethidium bromide.

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. and by Naruse et al.

**Detection of aPL**

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

For the determination of anti-ß2-glycoprotein I antibody (aß2GPI), ELISA was carried out as previously described by using gamma irradiated plates (Maxisorp), 80 µl of 10 µg/ml human purified prothrombin (Diagnostica Stago, Asneres, France) as an antigen, and 0.5% gelatin as a blocking reagent, as previously described.

Antiprothrombin antibody (aPT) was tested by ELISA using gamma irradiated plates (Maxisorp), 80 µl of 10 µg/ml human purified prothrombin (Diagnostica Stago, Choshi, Japan) in phosphate buffered saline (PBS) as an antigen, and 3% porcine skin gelatin (Sigma, St Louis, Missouri, USA) as a blocking reagent.

The tests for lupus anticoagulant (LA) were carried out according to the guidelines of the Scientific and Standardization Subcommittee on LA 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.

**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 χ² tests with the possibility of linkage disequilibrium, the delta (Δ) values—that is, the correlation coefficients in the 2x2 tables—were also calculated.

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

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

n = number of alleles in each group.

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

<table>
<thead>
<tr>
<th>Allele</th>
<th>aCL (n = 134)</th>
<th>aß2GPI (n = 46)</th>
<th>aPT (n = 46)</th>
<th>LA (n = 124)</th>
<th>Controls (n = 218)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMA*0101</td>
<td>76.1%</td>
<td>76.1%</td>
<td>83.3%</td>
<td>81.8%</td>
<td>85.8%</td>
</tr>
<tr>
<td>*0102</td>
<td>20.1</td>
<td>19.6</td>
<td>16.7</td>
<td>18.2</td>
<td>8.3</td>
</tr>
<tr>
<td>*0103</td>
<td>2.2</td>
<td>2.2</td>
<td>0.0</td>
<td>0.0</td>
<td>3.2</td>
</tr>
<tr>
<td>*0104</td>
<td>1.5</td>
<td>2.2</td>
<td>0.0</td>
<td>0.0</td>
<td>2.8</td>
</tr>
<tr>
<td>DMB*0101</td>
<td>75.4</td>
<td>75.4</td>
<td>72.2</td>
<td>75.0</td>
<td>69.7</td>
</tr>
<tr>
<td>*0102</td>
<td>1.5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>2.3</td>
</tr>
<tr>
<td>*0103</td>
<td>23.4</td>
<td>23.4</td>
<td>27.3</td>
<td>25.0</td>
<td>23.4</td>
</tr>
<tr>
<td>*0104</td>
<td>0.7</td>
<td>2.1</td>
<td>0.0</td>
<td>0.0</td>
<td>2.3</td>
</tr>
<tr>
<td>*0105</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

n = number of alleles in patients with positive antibodies.

Δp = 0.012, Δp = 0.007 v controls by 4x2 χ² test.

Δp = 0.002 (pc = 0.008), Δp = 0.001 (pc = 0.004) v controls by 2x2 χ² test.

aß2GPI, anti-ß2GPI antibody; aCL, anticardiolipin antibody; aPT, anti-prothrombin antibody; LA, lupus anticoagulant.

www.annrheumdis.com
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, 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, 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. The presence of polymorphisms in DM genes 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, insulin dependent diabetes, juvenile dermatomyositis, rheumatoid arthritis, and SLE. 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 dermatomyositis and the increased frequency of DMA*0103 or DMB*0101 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. 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*0101-DQA1*0101-DQB1*0501 haplotype. 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*0701-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,

| Allele | aCL +/− (n = 70/94) | aPT +/− (n = 110/120) | LA +/− (n = 58/90) |
|--------|------------------|------------------|----------------|---|
| DMA*0101 | 75.7%/86.2% | 18.8%/14.2% | 75.9%/86.7% |
| *0102 | 21.4%/48.8 | 20.0%/13.6 | 24.1%/19.5 |
| *0103 | 1.2/2.5 | 0.0/2.5 | 0.0/3.3 |
| *0104 | 1.2/2.0 | 1.2/2.1 | 0.0/0.9 |

n = number of alleles in patients with positive aPL. aPL, antiphospholipid antibodies; aCL, anticardiolipin antibody; aPT, anti-prothrombin antibody; LA, lupus anticoagulant.
neither DRB1*0701 nor DQB1*0303 was significantly associated with APS (the discrepancy from our previous report 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.

ACKNOWLEDGMENTS
This work was supported by Lupus UK. We are grateful to Mrs Kiran Parmar for performing the tests for lupus anticoagulant.

Authors’ affiliations
M L Sanchez, K Katsumata, T Atsumi, F I Romero, M L Bertolaccini, Parmar for performing the tests for lupus anticoagulant. This work was supported by Lupus UK. We are grateful to Mrs Kiran

REFERENCES
27 Djillali Saïd I, Beni V, Daniel S, Assam R, Bosh JF, Caillaux-Zuman S. Linkage disequilibrium between HLA class II (DR, DQ, DP) and antigen processing (UMP, TAP, DM) genes of the major histocompatibility complex. Tissue Antigens 1999;48:87–92.