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A study of HLA-DR and -DQ alleles in 588 patients and 562 controls confirms that HLA-DRB1*03 is associated with recurrent miscarriage

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BACKGROUND: Previous studies have demonstrated an association between recurrent miscarriage (RM) and the maternal HLA-DRB1*01 and -DRB1*03 alleles. The primary aim of the present study was to confirm or reject the hypothesis about this association in a larger case–control study. METHODS: HLA-DRB1, -DQA1 and -DQB1 genotyping was carried out by the PCR–sequence-specific primer (SSP) method in 354 patients with unexplained RM and 202 fertile controls. These results were combined with the results from a previous study of 234 RM patients and 360 controls. RESULTS: The prevalence of patients with HLA-DRB1*03 was significantly increased compared with controls [odds ratio (OR) = 1.4, 95% confidence interval (CI) = 1.1–1.9, *P* = 0.01, *P* corrected for the number of comparisons (*P*c) = 0.02]. In patients with at least four previous miscarriages or with secondary RM, the association became even stronger (OR = 1.8, 95% CI = 1.3–2.5, *P* = 0.0005, *P*c = 0.004; and OR = 1.8, 95% CI = 1.3–2.5, *P* = 0.0007, *P*c = 0.006, respectively). There was no significant differerence between patients and controls with regard to HLA-DRB1*01. CONCLUSION: The HLA-DRB1*03 allele or genes in linkage disequilibrium with it confer susceptibility to RM.

Key words: abortion/HLA antigens/HLA class II/immunology/recurrent miscarriage

Introduction

Recurrent miscarriage (RM), defined as three or more consecutive miscarriages, affects ~1% of the female population. The pathogenesis of most cases remains unknown, but several studies have indicated that the majority of these cases are caused by immunological disturbances (Christiansen, 1996; Coulam, 2000).

If immunological factors influence the risk of RM, the HLA system might play a role. Previous studies have focused on the theory of increased HLA compatibility between spouses as a cause of inappropriate immune recognition of the trophoblast and subsequent miscarriage (Coulam *et al.*, 1987; Ho *et al.*, 1990), but the majority of studies could not demonstrate increased sharing of HLA antigens (Oksenberg *et al.*, 1984; Christiansen *et al.*, 1989; Orgad *et al.*, 2000), and in prospective studies increased HLA sharing exhibited no prognostic impact on future pregnancy outcome (Recurrent Miscarriage Immunotherapy Trialists Group, 1994).

Numerous studies have reported that RM patients more often than fertile controls are positive for a series of autoantibodies, especially antiphospholipid antibodies (Branch, 1998). If systemic or localized autoimmunity is involved in the pathogenesis of unexplained RM, an association with certain HLA class II alleles would be expected, as almost all autoimmune disorders are associated with genes in these loci (Klein and Sato, 2000). This theory is supported by the finding that HLA-DRB1*03 is associated with positivity for anticardiolipin antibodies and antinuclear antibodies in RM patients (Christiansen *et al.*, 1998). During the last decade, a series of studies by the authors' group have focused on the association between HLA class II alleles and unexplained RM. In a case–control study using the restriction fragment length polymorphism (RFLP) technique, the frequencies of the HLA-DRB1*01 and -DRB1*03 allogenotypes were significantly increased among patients with at least four previous miscarriages, and it increased significantly with the number of previous fetal losses (Christiansen *et al.*, 1994). In a prospective study, RM patients with these allogenotypes had a higher miscarriage rate in their next pregnancy compared with other patients (Christiansen *et al.*, 1993), and in a family study, female relatives of RM patients had a significantly higher risk of miscarriage if they had a HLA-DRB1*01 or -DRB1*03 allogenotype compared with corresponding women without these types (Christiansen *et al.*, 1995). Finally, a meta-analysis including 18 previous studies exhibited a significantly increased odds ratio (OR) for being DRB1*01 antigen positive among RM patients of

Table I. Numbers (%) of patients with recurrent miscarriage and controls positive for each HLA-DRB1 allele

DRB1 allele	Study I		Study II		OR, study II (95% CI)	Combined OR study I and II (95% CI)
	Patients n = 234	Controls n = 360	Patients n = 354	Controls n = 202		
01	52 (22.2)	61 (16.9)	70 (19.8)	47 (23.3)	0.8 (0.5–1.2)	1.1 (0.8–1.4)
03	66 (28.2)	76 (21.1)	94 (26.6)	42 (20.8)	1.4 (0.9–2.1)	1.4 (1.1–1.9)[a]
04	87 (37.2)	123 (34.2)	136 (38.4)	61 (30.2)	1.4 (1.0–2.1)[b]	1.3 (1.0–1.6)[c]
07	33 (14.1)	63 (17.5)	60 (16.9)	36 (17.8)	0.9 (0.6–1.5)	0.9 (0.6–1.2)
08	14 (6.0)	36 (10.0)	29 (8.2)	18 (8.9)	0.9 (0.5–1.7)	0.7 (0.5–1.1)
09	2 (0.9)	5 (1.4)	3 (0.8)	5 (2.5)	0.3 (0.1–1.4)	0.4 (0.2–1.3)
10	8 (3.4)	4 (1.1)	7 (2.0)	1 (0.5)	4.1 (0.5–33)	3.4 (1.2–9.8)[d]
11	45 (19.2)	63 (17.5)	53 (15.0)	30 (14.9)	1.0 (0.6–1.6)	1.1 (0.8–1.5)
12	2 (0.9)	6 (1.7)	12 (3.4)	9 (4.5)	0.8 (0.3–1.8)	0.7 (0.3–1.5)
13	61 (26.1)	98 (27.2)	74 (20.9)	63 (31.2)	0.6 (0.4–0.9)[e]	0.8 (0.6–1.0)[f]
14	10 (4.3)	14 (3.9)	10 (2.8)	13 (6.4)	0.4 (0.2–1.0)[g]	0.7 (0.4–1.3)
15	58 (24.8)	112 (31.1)	118 (33.3)	51 (25.2)	1.5 (1.0–2.2)	–
16	2 (0.9)	5 (1.4)	7 (2.0)	4 (2.0)	0.9 (0.2–3.1)	0.8 (0.3–2.0)

[a]P = 0.01, Pc = 0.02; [b]P = 0.05, Pc = 0.65; [c]P = 0.06, Pc = 0.78; [d]P = 0.02, Pc = 0.26; [e]P = 0.02, Pc = 0.26; [f]P = 0.04, Pc = 0.52; [g]P = 0.04, Pc = 0.52.
The combined OR was calculated when the test for heterogeneity between study I and II was >0.05.

Caucasian ethnic origin compared with controls, whereas there was no difference between patients and controls with regard to the HLA-DRB1*03 allele (Christiansen *et al.*, 1999).

The aims of the present study were to confirm or reject the hypothesis of an association between RM and the HLA-DRB1*01 and -DRB1*03 alleles in a larger study, to separate the contribution of each of the alleles and to investigate interactions of importance with other HLA-DRB1, -DQA1 and -DQB1 alleles.

Materials and methods

Study I refers to a previously published case–control study (Christiansen *et al.*, 1994). Study II comprised a new group of patients, included between 1994 and 2002, and a new control group.

Patients

The new patient group (study II) comprised 354 Caucasian women with RM, who had been admitted to the authors' clinic from all over Denmark for investigation and treatment. All patients had a history of three or more miscarriages, confirmed by hospital records. None of the patients had uterine abnormalities found by hysteroscopy or hysterosalpingography, and all patients and their husbands had normal karyotypes. All women were regularly menstruating with a cycle length of <35 days and all had normal plasma thyroxin levels. Of these patients, 195 (55%) had had four or more previous miscarriages. Primary RM was found in 212 patients (60%) and 142 patients (40%) had secondary RM, with at least one birth after 28 weeks of gestation preceding RM. The patients had a median age of 32 years (range 20–45) and had had a median number of four miscarriages (range 3–10).

The patient group of study I comprised 234 Caucasian women with RM who had undergone the same investigation programme as the new group.

In the combined patient group of 588 women, 292 (50%) had had four or more previous miscarriages, and 250 (43%) had secondary RM.

Controls

The control group of study II comprised 202 Caucasian individuals from couples with two or more children (median 2, range 2–4) and a history of no miscarriages. Most control couples (71%) were contacted in the maternity ward after their last delivery. The remainder were staff members and their spouses.

The control group of study I mainly comprised 360 healthy, unrelated, Caucasian Danish blood donors of both sexes.

PCR-SSP typing

All patients and controls in study II were genotyped using a previously described PCR–sequence-specific primer (SSP) technique (Olerup *et al.*, 1992, 1993). In short, DNA was isolated from nucleated blood cells. Sequence-specific amplification was carried out using 21 primers for the DRB1 locus and a primer for each of the DRB3, DRB4 and DRB5 loci, 21 primers for the DQA1 locus and eight for the DQB1 locus (Dynal Allsets, Oslo, Norway). The PCR was carried out using a DNA Thermal Cycler (Perkin-Elmer Gene Amp 9600). After agarose gel electrophoresis, the genotype for each patient was interpreted, using the SSP-tool program (Dynal Biotech, Oslo, Norway).

RFLP typing

Allelic *Taq*I DRB1, DQA1 and DQB1 RFLP typings were carried out in patients and controls in study I, as previously described (Christiansen *et al.*, 1994).

Ethical aspects

Informed consent was obtained from all patients and controls, and the study protocol was approved by the local ethics committee. The study was considered in agreement with the Declaration of Helsinki regarding research in humans.

Statistical analysis

For comparisons between patients and controls in each study (study I and II), the OR was calculated by Haldane's modification of Woolf's method (Haldane, 1955). Whether OR values differed statistically significantly from unity was estimated by the χ^2 test. As studies I and II were different with regard to the control groups and to the laboratory methods, the combined ORs were calculated using a meta-analysis program [Review Manager (RevMan) Version 4.2 for Windows. Oxford, UK: The Cochrane Collaboration, 2002]. This program weighs the included studies not only according to the total number of individuals in each study, but also according to the number of patients

Table II. Numbers (%) of patients with recurrent miscarriage and controls positive for each HLA-DQA1 allele

DQA1 allele	Study I		Study II		OR, study II (95% CI)	Combined OR study I and II (95% CI)
	Patients n = 234	Controls n = 360	Patients n = 354	Controls n = 202		
0101	65 (27.8)	75 (20.8)	70 (19.8)	50 (24.8)	0.8 (0.5–1.1)	–
0102	87 (37.2)	148 (41.1)	139 (39.3)	72 (35.6)	1.2 (0.8–1.7)	1.0 (0.8–1.3)
0103	31 (13.2)	53 (14.7)	44 (12.4)	43 (21.3)	0.5 (0.3–0.8)[a]	0.7 (0.5–1.0)[b]
0104	0	0	10 (2.8)	10 (5.0)	0.6 (0.2–1.4)	–
0105	0	0	4 (1.1)	0	5.2 (0.3–97)	–
0201	33 (14.1)	64 (17.8)	59 (16.7)	36 (17.8)	0.9 (0.6–1.5)	0.8 (0.6–1.2)
03	88 (37.6)	125 (34.7)	140 (39.5)	66 (32.6)	1.4 (0.9–1.9)	1.2 (1.0–1.6)
0401	11 (4.7)	32 (8.9)	25 (7.1)	18 (8.9)	0.8 (0.4–1.5)	0.6 (0.4–1.0)[c]
05	110 (47.0)	141 (39.2)	148 (41.8)	79 (39.1)	1.1 (0.8–1.6)	1.3 (1.0–1.6)[d]
0601	2 (0.9)	4 (1.1)	2 (0.6)	0	2.9 (0.1–60)	1.1 (0.3–4.5)

[a]$P < 0.01$, $Pc = 0.7$; [b]$P = 0.02$, $Pc = 0.2$; [c]$P = 0.04$, $Pc = 0.4$; [d]$P = 0.07$, $Pc = 0.70$.
The combined OR was calculated when the test for heterogeneity between study I and II was >0.05.

Table III. Numbers (%) of patients with recurrent miscarriage and controls positive for each HLA-DQB1 allele

DQB1 allele	Study I		Study II		OR, study II (95% CI)	Combined OR study I and II (95% CI)
	Patients n = 234	Controls n = 360	Patients n = 354	Controls n = 202		
0201	80 (34.2)	120 (33.3)	124 (35.0)	64 (31.7)	1.2 (0.8–1.7)	1.1 (0.9–1.4)
0301	89 (38.0)	126 (35.0)	105 (29.7)	60 (29.7)	1.0 (0.7–1.5)	1.1 (0.8–1.4)
0302	63 (26.9)	85 (23.6)	92 (26.0)	38 (18.8)	1.5 (1.0–2.3)[a]	1.3 (1.0–1.8)[b]
0303	12 (5.1)	22 (6.2)	24 (6.8)	14 (6.9)	1.0 (0.5–1.9)	0.9 (0.6–1.5)
0304	0	0	3 (0.8)	0	4.0 (0.2–78)	–
04	12 (5.1)	32 (8.9)	25 (7.1)	18 (8.9)	0.8 (0.4–1.5)	0.7 (0.4–1.0)
0501	58 (24.8)	60 (16.7)	73 (20.6)	47 (23.3)	0.9 (0.6–1.3)	–
0502	2 (0.9)	5 (1.4)	6 (1.7)	4 (2.0)	0.9 (0.2–3.1)	0.8 (0.3–2.0)
0503	10 (4.3)	14 (3.9)	10 (2.8)	10 (5.0)	0.6 (0.2–1.4)	0.8 (0.4–1.5)
0601	3 (1.3)	6 (1.7)	3 (0.8)	4 (2.0)	0.4 (0.1–1.9)	0.6 (0.2–1.7)
0602	57 (24.4)	11 (30.8)	111 (31.4)	50 (24.8)	1.4 (0.9–2.1)	–
0603/0604	58 (24.8)	89 (24.7)	72 (20.3)	62 (30.7)	0.6 (0.4–0.9)[c]	0.8 (0.6–1.0)

[a]$P = 0.05$, $Pc = 0.6$; [b]$P = 0.05$, $Pc = 0.6$; [c]$P < 0.01$, $Pc = 0.08$.
The combined OR was calculated when the test for heterogeneity between study I and II was >0.05.

positive for the HLA allele in question, a method which has been recommended (Svejgaard *et al.*, 1974). The *P*-value for heterogeneity between the included studies is also tested in the program. This should be >0.05 to allow a combined OR to be calculated.

In all tables of this study, the phenotype frequencies (frequency of individuals carrying the HLA allele) between patients and controls were compared, since this is generally recommended in studies of disease associations (Svejgaard and Ryder, 1994).

The test for increasing or decreasing trend was performed by Spearman's ρ.

A *P*-value ⩽0.05 was considered significant. *P*-values were corrected (*Pc*-values) according to previous recommendations (Svejgaard and Ryder, 1994): as the two alleles HLA-DRB1*01 and -DRB1*03 were subjected to an *a priori* hypothesis, their *P*-values were multiplied by two. For all other HLA-DRB1 or -DQ alleles, the *P*-values were corrected by multiplication by the number of investigated alleles in each table. For analyses of subgroups of patients, these *P*-values were corrected further by multiplication by the number of investigated subgroups.

Results

The *P*-value for heterogeneity between study I and II was for the great majority of alleles >0.05, and for these alleles a combined OR for study I and II was calculated. For the alleles HLA-DRB1*15, DQA1*0101, DQB1*0501 and DQB1*0602, the *P*-values for heterogeneity were <0.05, and for DQA1*0104, DQA1*0105 and DQB1*0304 the test was not applicable, as these alleles were not found in any of the patients or controls in study I. For HLA-DRB1*03, the *P*-value for heterogeneity between the studies was as high as 0.82, indicating that the studies were easily combinable concerning this allele.

As expected, the distribution of HLA-DRB1 phenotypes was similar in male and female controls. As for HLA-DRB1*03, this phenotype was found in 21.5% of male and 20.5% of female controls, respectively, in the combined control group.

For study II alone, no differences between patients and controls were significant after correction for multiple comparisons (Tables I–III).

In the combined studies, RM patients with the HLA-DRB1*03 allele were found with a significantly increased frequency compared with the controls [OR = 1.4, 95% confidence interval (CI) 1.1–1.9, $P = 0.01$, $Pc = 0.02$; Table I]. Patients with HLA-DRB1*10 were also found with increased frequency (OR = 3.4, 95% CI 1.2–9.8, $P = 0.02$,

Table IV. Numbers (%) of patients with at least four miscarriages (4+ RM), patients with secondary recurrent miscarriage (Sec. RM) and controls, positive for suggested HLA-DRB1 susceptibility alleles

Allele	4+ RM n = 292	Sec. RM n = 250	Controls n = 562	OR_1 (95% CI)	OR_2 (95% CI)
DRB1*01	66 (22.6)	48 (19.2)	108 (19.2)	1.2 (0.8–1.7)	1.0 (0.6–1.4)
DRB1*03	92 (31.5)	81 (32.4)	118 (21.0)	1.8 (1.3–2.5)[a]	1.8 (1.3–2.5)[b]
DRB1*04	106(36.3)	91 (36.4)	184 (32.7)	1.2 (0.8–1.6)	1.2 (0.9–1.7)
DRB1*10	9 (3.1)	8 (3.2)	5 (0.9)	3.9 (1.1–13.5)[c]	4.0 (1.2–13.2)[d]

OR_1 - RM patients with at least four miscarriages versus controls; OR_2 = secondary RM patients versus controls.
[a]P = 0.0005, Pc = 0.004; [b]P = 0.0007, Pc = 0.006; [c]P = 0.03, Pc = 0.63; [d]P = 0.02, Pc = 0.42.

Table V. Numbers (%) of recurrent miscarriage patients positive for HLA-DRB1*03, according to number of previous miscarriages

No. of miscarriages	HLA-DRB1*03/total	%
3	68/296	23.0
4	41/149	27.5
⩾5	51/143	35.7

P = 0.01, Pc = 0.02 (test for increasing trend).

Table VI. OR for genotypic combinations of HLA-DRB1*03/DQB1*0501 and HLA-DRB1*03/HLA-DRB1*04 in patients with RM, patients with at least four miscarriages (4+ RM) and patients with secondary RM

	DRB1*03/DQB1*0501 OR (95% CI)	DRB1*03/DRB1*04 OR (95% CI)
All RM patients	1.7 (0.8–4.0)	1.9 (1.1–3.4)[a]
4+ RM patients	2.8 (1.2–6.8)[b]	1.9 (1.0–3.8)
Secondary RM patients	2.0 (0.7–5.2)	2.0 (1.0–3.9)

[a]P = 0.03, Pc = 0.18; [b]P = 0.02, Pc = 0.12.

Pc = 0.26), although the difference was not significant after correction. Due to a lower frequency of HLA-DRB1*04 in the control group of study II, patients with this allele were found with an increased frequency in the combined studies (OR =1.3, 95% CI = 1.0–1.6, P = 0.06, Pc = 0.78), although the difference was not significant.

The frequency of patients with the HLA-DQA1*05 allele was increased, although the difference was not significant (OR = 1.3, 95% CI = 1.0–1.6, P = 0.07, Pc = 0.70; Table II).

Patients with the HLA-DQB1*0302 allele were found with a significantly increased frequency, although not after correction (OR = 1.3, 95% CI = 1.0–1.8, P = 0.05, Pc = 0.60; Table III).

The associations with the HLA-DRB1*03 allele were stronger in subgroups of patients with at least four miscarriages (OR = 1.8, 95% CI = 1.3–2.5, P = 0.0005, Pc = 0.004) and in patients with secondary RM (OR = 1.8, 95% CI = 1.3–2.5, P = 0.0007, Pc = 0.006) than the corresponding associations among all RM patients. The same trend was observed for the HLA-DRB1*10 allele, but not for the HLA-DRB1*01 and HLA-DRB1*04 alleles (Table IV).

There was an overlap of 163 patients who had had at least four miscarriages and had secondary RM. For the HLA-DRB1*03 allele, it was calculated, that the ORs for RM in the subset of patients with only three miscarriages were 0.9 and 1.6 for patients with primary and secondary RM, respectively. For the subgroup of patients with at least four miscarriages, the corresponding ORs were 1.4 and 2.0, respectively.

The frequencies of the HLA-DRB1*03 allele in subgroups of patients with three, four or ⩾5 previous miscarriages increased significantly with the number of miscarriages (P = 0.01, Pc = 0.02; Table V).

Table VI shows genotypic combinations of relevant HLA-DRB1 and -DQB1 alleles and their associations with RM. The HLA-DRB1*03/DQB1*0501 genotype conferred a high OR for suffering four or more miscarriages, although this was not significant after correction (OR = 2.8, P = 0.02, Pc = 0.12), and the HLA-DRB1*03/DRB1*04 genotype tended to confer a higher OR for RM than the HLA-DRB1*03 allele alone.

The ORs for RM for the DQA1*0501/*0505 alleles and for the DQB1*0201 allele were calculated, stratified according to being together with HLA-DRB1*03 (on the same haplotype in the *cis* position or on the other haplotype in the *trans* position), or being in genotypes without HLA-DRB1*03. For the DQA1*0501/*0505 alleles, the OR was 1.4 (95% CI = 0.2–9.8, P = 0.76) in individuals positive for HLA-DRB1*03 and 1.1 (95% CI = 0.8–1.5, P = 0.54) in HLA-DRB1*03-negative individuals. For the DQB1*0201 allele, the ORs were 1.1 (95% CI = 0.3–7.7, P = 0.95) and 0.7 (95% CI = 0.5–1.1, P = 0.17), respectively.

Discussion

The present study is the largest case–control study of the prevalence of HLA alleles in women with RM published so far. A meta-analysis has been published previously, including 18 case–control studies of HLA-DR frequencies in Caucasian RM patients (Christiansen *et al.*, 1999). Only a few further studies have been published (Bellingard *et al.*, 1995; Sbracia *et al.*, 1996; Pennesi *et al.*, 1998; Orgad *et al.*, 2000). Significant association (after correction) with RM has been found for HLA-DR5 (Gerencer and Kastelan, 1983; Smith *et al.*, 1989) and for HLA-DRB1*01 (McIntyre *et al.*, 1984; Beer *et al.*, 1985), which was also found significantly associated in the meta-analysis (Christiansen *et al.*, 1999), but the majority of previous studies found no susceptible alleles for RM. However, most of the studies were small, only two studies used a DNA-based method (Laitinen *et al.*, 1993; Christiansen *et al.*, 1994) and in eight studies patients with autoantibodies were excluded, which may have biased the results regarding HLA-DRB1*03, as this allele predisposes to formation of antiphospholipid antibodies (Christiansen *et al.*, 1998). Associations

between RM and other HLA-DRB1 alleles have been found in Japanese studies (Sasaki *et al.*, 1997; Takakuwa *et al.*, 2003) but, due to the great variation of allele frequencies between different ethnic groups, these results may not apply to Caucasian populations.

In the present study, two study groups were combined. The frequencies of some alleles differed between the old and new groups of patients and controls, respectively, (Table I–III). Whereas the patients in the two studies have been recruited under identical selection criteria, the control groups, although recruited from the same local area, had different origins: in study I, most controls were blood donors, and therefore selected to be healthy, but with an unknown reproductive history, whereas in study II the controls were couples with proven normal fecundity, and otherwise may be more representative of the normal population. However, couples with normal fecundity are generally healthy and, due to the low prevalence of RM in the general population, it can be expected that most blood donors have normal fecundity. The variation in the allele frequencies could be attributed to chance, since the test for heterogeneity for most alleles justified the combination of the two studies.

The present study confirmed our prior hypothesis that the HLA-DRB1*03 allele confers susceptibility to RM, since patients with this allele were found with a significantly increased frequency compared with controls before and after adjustment for multiple comparisons (Table I). The adjustment of this *P*-value was carried out according to the *a priori* hypothesis in our protocol that HLA-DRB1*01 and -DRB1*03 would be found with increased prevalence in the RM patients. This prior hypothesis was based on results from our previous case–control study (Christiansen *et al.*, 1994), a prospective study (Christiansen *et al.*, 1993) and a family study (Christiansen *et al.*, 1995). In the latter two studies, the significance of the HLA-DRB1*01 and -DRB1*03 alleles was investigated together *en bloc*, and the main purpose of the present study was to separate the contribution of each of the alleles to the susceptibility to RM.

In subgroups of patients with severe RM (at least four miscarriages) or with secondary RM, the associations with HLA-DRB1*03 became highly significant (Table IV), and were still highly significant after correction of the *P*-value for the number of subgroup analyses. Since there was an overlap of 163 patients who had both severe and secondary RM, the stronger association between HLA-DRB1*03 and secondary RM could be caused by a higher number of miscarriages in this subgroup. However, it was calculated that the ORs were higher for patients with secondary RM compared with those with primary RM in the subgroup of patients with only three miscarriages (0.9 and 1.6, respectively) as well as in the subgroup with at least four miscarriages (1.4 and 2.0, respectively). The frequency of the HLA-DRB1*03 allele increased significantly with increasing number of previous miscarriages (Table V), which might indicate a causal relationship.

Surprisingly, the study could not confirm that HLA-DRB1*01 confers susceptibility to RM since, in the new sample of patients and controls (study II), the prevalence of this allele was lower in patients than in controls (Table I). In the control group of study II, the prevalence of individuals positive for HLA-DR1 was >6% higher than in study I. Some of this variation could be attributed to chance, since the test for heterogeneity between studies did not find them significantly different, or it might be explained by the different origin of the control groups, as previously discussed. On the other hand, study II gave further support for the theory that HLA-DRB1*10 or linked HLA-DQ alleles are risk alleles for RM. In study I, this allele was associated with RM exhibiting an OR of 3.2, which was, however, not statistically significantly increased due to the rarity of the allele. In study II, a similar OR was found, resulting in a combined OR of 3.4 that was significantly increased before adjustment for multiple comparisons. In our previous publication (Christiansen *et al.*, 1994), we put forward the hypothesis that the DQB1*0501 allele rather than the HLA-DRB1*01 and -DRB1*10 alleles was the susceptibility allele for RM since this DQB1 allele is in strong linkage disequilibrium with both HLA-DRB1*01 and -DRB1*10 and, in our population, found on almost all haplotypes with these HLA-DRB1 alleles. The findings in the present study concerning HLA-DRB1*01 and HLA-DRB1*10 weaken this hypothesis, and the results shown in Table VI provide no unambiguous answer to the question of whether HLA-DQB1*0501 found together with HLA-DRB1*03 results in a genotype which confers a higher risk for RM than HLA-DRB1*03 on its own.

In Table VI the question was addressed of whether HLA-DRB1*03 in combination with HLA-DQB1*0501 or with HLA-DRB1*04 confers a higher susceptibility to RM than HLA-DRB1*03 alone. As mentioned above, it was hypothesized in a previous study (Christiansen *et al.*, 1994) that HLA-DQB1*0501 is associated with RM. The finding of a higher OR (2.8) for the DRB1*03/DQB1*0501 combination than for HLA-DRB1*03 alone (1.8) in patients with four or more miscarriages might support this hypothesis. The frequency of HLA-DRB1*04 was increased in RM patients compared with controls (Table VI), and since HLA-DRB1*03 and -DRB1*04 have a synergistic effect on the predisposition to an autoimmune disease, insulin-dependent diabetes mellitus (Noble *et al.*, 1996), this might also apply to the susceptibility to RM. The present results indeed indicate that the HLA-DRB1*03/DRB1*04 genotype tended to confer a higher OR for RM than the HLA-DRB1*03 allele alone (Tables IV and VI).

The frequency of some alleles was significantly decreased in patients compared with controls (Tables I–III), although not after correction for multiple comparisons. This can be explained by the relatively higher frequency of other alleles in the patient group, and little emphasis has been put on this finding.

Strong linkage disequilibrium exists between HLA-DRB1, -DQA1 and -DQB1 alleles, meaning that some alleles in these loci are found together on the same haplotype much more frequently than expected by chance alone. An example is HLA-DRB1*03, which in Caucasian populations in practice always implies the presence of DQA1*0501 and DQB1*0201 on the same haplotype. Therefore, an association between an allele,

e.g. HLA-DRB1*03, and a disorder could be secondary to an association between the DQA1*0501 or DBB1*0201 allele. A method has been recommended for calculating which allele among several candidates found in linkage disequilibrium with each other exhibits the strongest association with a disorder (Svejgaard and Ryder, 1994). These calculations were done for the HLA-DRB1*03, DQA1*0501, DQB1*0201 haplotype, as the demonstrated association between RM and HLA-DRB1*03 in principle could be secondary to associations with DQA1*0501, which is found with increased frequency (although not significantly) in patients, or with DQB1*0201. When the associations of the DQA1*0501 and DQB1*0201 alleles with RM are stratified in patients and controls according to positivity or negativity for HLA-DRB1*03, the ORs in HLA-DRB1*03-negative patients (1.1 and 0.7 for the DQA1*0501 and DQB1*0201 alleles, respectively) tended to be lower than the OR of 1.4 for HLA-DRB1*03, indicating that it is the HLA-DRB1*03 allele, rather than the DQA1 and DQB1 alleles found in linkage disequilibrium with it, that exhibits the primary association with RM. However, some reservations must be noted for these calculations, since no deviations were significant and certain combinations of alleles were not observed, either in patients or in controls.

The pathophysiolocigal explanation for the association between HLA-DRB1*03 and RM is still not clear. A possible model might be that HLA-DRB1*03 predisposes to an autoreactive immune response against one or more trophoblast antigens: HLA-DRB1*03 molecules on maternal antigen-presenting cells may present trophoblast-derived peptides to maternal autoreactive T cells. As another possible explanation, the association might be caused by a close linkage between the HLA-DRB1 genes and the genes for tumour necrosis factor (TNF), since it has been reported that HLA-DRB1*03 and -DRB1*04 are in linkage disequilibrium with alleles predisposing to *in vitro* hypersecretion of TNF-α (Pociot *et al.*, 1993), which in murine and human *in vitro* studies has exhibited embryotoxic or trophoblast-inhibiting activity (Raghupathy, 1997). A third explanation may be that HLA-DRB1*03 is in linkage disequilibrium with HLA-G alleles associated with RM (Hviid *et al.*, 2002). HLA-G is of particular interest with respect to the reproductive processes, as it is the dominant HLA antigen on the human trophoblast.

In conclusion, it was confirmed in the present study that HLA-DRB1*03 confers susceptibility to RM, and the association is probably not caused by the DQA1 and DQB1 alleles found in linkage disequilibrium with it. A genotypic combination of HLA-DRB1*03 with -DRB1*04 or with -DQB1*0501 might increase the susceptibility to RM, but this finding must be confirmed in further studies. The results of the present study could not confirm the hypothesis of an association between HLA-DRB1*01 and RM. Larger studies are needed if the main susceptibility gene sequences are to be identified with certainty. However, the results up until now indicate that RM is a multifactorial condition, and that HLA-DRB1*03, and perhaps other class II antigens, does not act as a sole factor to cause miscarriage, but increases the risk of fetal loss when found in conjunction with other immunological (Kruse *et al.*, 2002) or haemophilic (Rey *et al.*, 2003) disturbances.

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