HLA class II DR, DQ, and DP restriction fragment length polymorphisms in rheumatoid arthritis

W M HOWELL,¹ P REEVANS,¹ P J WILSON,² M I D CAWLEY,³ AND J L SMITH¹

From the ¹Wessex Regional Immunology Service, Southampton General Hospital; the ²Tissue Typing Laboratory, Wessex Regional Transfusion Centre, Southampton; and the ³Rheumatology Unit, Southampton General Hospital, Southampton

SUMMARY HLA class II restriction fragment length polymorphisms (RFLPs) were studied in 43 individuals with established seropositive rheumatoid arthritis (RA) and in a group of healthy controls. All patients and controls were tissue typed for HLA-A, B, and DR antigens. Rapid, initial screening for RA associated RFLPs was conducted by pooling DNA samples from 11 HLA-DR4 positive patients with RA and comparing the RFLP patterns with those seen in a pool of DNA samples drawn from 11 HLA-DR4 positive healthy controls. Candidate RA associated RFLPs were examined in our full panel of patients with RA and controls. In most cases the RFLPs detected showed no significant association with RA. An exception was a 13.0 kb *Dra*I DQ β associated RFLP, which, when HLA-DR4 positive patients with RA and controls were considered alone, showed a weak positive association with susceptibility to RA. This RFLP was not associated with known DR, DQ, or Dw specificities. These results show a distinct paucity of class II RA associated RFLPs but may indicate a role for DQ β genetic variation in the aetiology of RA.

Key words: rheumatoid arthritis susceptibility, HLA-DR4.

There is a well documented association between the HLA class II DR4 antigen and susceptibility to rheumatoid arthritis (RA) in most ethnic groups studied.¹ This is supported by evidence of haplotype sharing in affected sib pairs in multicase families with RA.^{2,3} This DR4 association with RA is weak (relative risk=4), however, and, furthermore, the DR4 antigenic specificity is heterogeneous at the cellular, serological, biochemical, and molecular genetic levels.^{4,5} Also, up to one third of patients with RA do not carry the DR4 antigen. Thus it is unlikely that DR4 represents the most precise class II genetic marker for RA susceptibility.

The HLA class II region is divided into three main subregions: DR, DQ, and DP. These DR, DQ, and DP antigens are expressed as highly polymorphic transmembrane $\alpha\beta$ heterodimers, with most polymorphism residing in the β chains. The DR antigens are known to be in strong linkage disequilibrium

with DQ, but less strongly with DP.⁶ Therefore an apparent association between DR4 and susceptibility to RA may reflect linkage disequilibrium between DR4 and an RA susceptibility gene elsewhere in the class II region. In consequence more precise HLA and RA associations may be detectable at the gene level using gene probing, Southern blotting, and restriction fragment length polymorphism (RFLP) analysis. In this study we examined DR β , DQ α , DQ β , DP α , and DP β RFLPs and their association with RA.

Patients, materials, and methods

PATIENTS AND CONTROLS

Forty three patients (26 female, 17 male) from Southampton with established classical or definite and seropositive RA without Felty's syndrome were included in this study. An identical number of healthy controls were selected from among laboratory staff, blood donors, and members of the local bone marrow donor panel.

CELL LINES

Eight HLA-DR4 homozygous typing cell lines of

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Correspondence to Dr W M Howell, Wessex Regional Immunology Service, Tenovus Research Laboratory, Southampton General Hospital, Tremona Rd, Southampton SO9 4XY, UK.

known HLA-Dw and DQ types were used to investigate possible RFLP correlations with the Dw4, Dw10, Dw13, Dw14, DKT2, and TA10 (DQw7) specificities associated with DR4.

HLA TISSUE TYPING

HLA tissue typing was performed by standard two stage National Institutes of Health microlymphocytotoxicity tests, using well defined sera for common Caucasian HLA-A, HLA-B, and HLA-DR1, 2, 3, 4, 5, w6, 7, and w8 antigens.

RESTRICTION ENZYMES

The full panel of 16 restriction enzymes used consisted of six enzymes cleaving at four base pair recognition sites—AluI, CfoI, HaeIII, MspI, RsaI, and TaqI—and 10 enzymes cleaving at six base pair recognition sites—ApaI, BamHI, BglII, DraI, EcoRV, HindIII, KpnI, PstI, PvuII, and XbaI.

PROBES

The following gene probes were used in the RFLP analysis: HLA-DR β pRTVI,⁷ HLA-DQ α pDCHI,⁸ HLA-DQ β pII- β -I,⁹ HLA-DP α pSB α ,¹⁰ and HLA-DP β .¹¹ The respective gene inserts were excised from low gelling temperature agarose gels after appropriate endonuclease restriction (generating 517 bp PstI DR β , 794 bp PstI DQ α , 620 bp AvaI DQ β , 1150 bp EcoRI DP α , and 1000 bp EcoRI/Hind III DP β gene fragments) and radiolabelled with α -[³²P]dCTP by the random hexanucleotide primer method.¹²

DNA PREPARATION, DIGESTION, SOUTHERN BLOTTING, AND RFLP ANALYSIS

High molecular weight DNA was prepared from peripheral blood or homozygous typing cell lines according to established procedures.⁷ DNA pools were constructed by mixing equal amounts of DNA from either 11 unrelated DR4 positive patients with RA or 11 unrelated DR4 positive controls, using the method of Arnheim *et al.*¹³ For each experiment 10 μ g of DNA was digested with 5 U/ μ g restriction enzyme under conditions specified by the manufacturers. After restriction, DNA fragments were separated by size via electrophoresis through 0.7% agarose gels and transferred to nylon (Hybond-N) filters according to the method of Southern.¹⁴ These filters were hybridised with ³²P labelled probes under standard conditions.⁷ Hybridising bands were visualised by autoradiography for 3–10 days at –70°C using Fuji-RX film. After autoradiography the probe was removed from filters by washing with 0.4 M NaOH at 45°C for 30 minutes, followed by 0.1 $\times$ SSC (0.15 M NaCl+0.015 M sodium citrate) 0.1% sodium dodecyl sulphate, 0.2 M trometamol (TRIS)-HCl pH 7.5 at 45°C for a further 30 minutes. Filters were subsequently sequentially rehybridised with further gene probes. Candidate RFLPs so detected were finally examined in an extended panel of patients with RA and healthy controls on an individual basis.

Results

All patients with RA entered into this study were

Table 1 Candidate rheumatoid arthritis (RA) associated restriction fragment length polymorphisms (RFLPs) detected by the DNA pooling method

Enzyme	Probe		
	DR β	DQ α	DQ β
AluI	–	–	–
ApaI	NT	–	NT
BamHI	–	–	–
BglII	–	–	NT
CfoI	–	–	–
DraI	–	–	–
EcoRV	NT	–	+(13.0 kb $\uparrow$, 6.8 kb $\uparrow$, 2.4 kb $\uparrow$)
HaeIII	NT	–	NT
HindIII	NT	–	NT
KpnI	–	–	NT
MspI	NT	+(6.3 kb $\uparrow$)	–
PstI	–	+(6.4 kb $\uparrow$)	–
PvuII	–	–	–
RsaI	–	+(1.9 kb $\uparrow$, 1.4 kb $\downarrow$)	–
TaqI	–	–	–
XbaI	–	–	–

–=no RFLP difference between RA and control pools; +=RFLP differences between RA and control pools; NT=not tested
 $\uparrow$ =enhancement of band in RA pool; $\downarrow$ =diminution of band in RA pool.

Fig. 1 Restriction fragment length polymorphism (RFLP) analysis of rheumatoid arthritis (RA) and control DNA pools (a) after restriction with *TaqI*, *RsaI*, *XbaI*, and *PstI* followed by hybridisation with the *DQα* probe and (b) after *DraI* restriction and *DQβ* hybridisation. R denotes RA DNA pool and C control pool. Numbers on the left denote molecular weight markers in kb while those on the right denote RFLPs in kb.

tissue typed for HLA-A, B, and DR antigens. Thirty of the 43 patients were DR4 positive, corresponding to a prevalence of 70%, while a smaller cohort of 11 patients was DR1 positive (26%). Four patients were DR1,4 heterozygotes, while the remaining six patients showed a range of DR antigenic specificities. Among the United Kingdom population at large the prevalence of DR4 is 36% and of DR1 is 16%.¹⁵ These results indicate primary DR4-RA and secondary DR1-RA associations in our patient population.

Candidate RA associated RFLPs were sought using our full panel of 16 restriction enzymes in conjunction with DRβ, DQα, and DQβ gene probes, using the DNA pooling technique. Several candidate RA associated RFLPs were detected by the criterion of relative band intensity in the RA pool digest compared with the control pool, for a particular restriction enzyme/probe combination. Table 1 gives the full results and Fig. 1 examples of some of these RFLPs (together with non-polymorphic RA/control pool pairs).

Probing with DRβ failed to show any candidate RA associated RFLPs. DQα probing, however, showed possible RA associated RFLPs with *MspI* (6.3 kb band enhanced in RA pool—data not shown), *PstI* (6.4 kb band enhanced in RA pool—Fig. 1a), and *RsaI* (1.9 kb band enhanced in RA pool and 1.4 kb band diminished—Fig. 1a). DQβ probing showed that 13.0, 6.8, and 2.4 kb *DraI* polymorphisms were enhanced in the RA pool, plus

evidence of further polymorphisms (Fig. 1b). All these candidate RFLPs were then examined in an extended panel of patients with RA and in healthy controls on an individual basis, irrespective of their DR type.

In this expanded analysis most of the candidate RFLPs showed no significant association with RA. For example, the 1.4 kb *RsaI* DQ α associated band was present in 8/17 (47%) patients with RA and 5/17 (29%) controls ($p=0.16$; Fisher's exact test). Also, the 6.4 kb *PstI* DQ α associated fragment was seen both in 2/17 (12%) patients with RA and 2/17 (12%) controls. Individual analysis of the *DraI* DQ β associated RFLPs showed a more complex situation with 7.2, 3.0, and 2.7 kb polymorphic bands in addition to the 13.0, 6.8, and 2.4 kb bands strongly detected as polymorphic in the pools. Other less frequent bands were also seen (Figs 2 and 3). Table 2 gives full results and statistical analysis of these *DraI* DQ β associated RFLPs. These results supported our initial finding of an increased prevalence of the 13.0 kb band among patients with RA as detected in the DNA pools. This association just failed to achieve significance at the 5% level when all patients with RA and controls, irrespective of their DR type, were considered. Among DR4 positive patients with RA and controls alone this 13.0 kb-RA association was apparently stronger, reaching significance at the 5% level. Nevertheless, the 13.0 kb band was detected among some non-DR4 patients with RA. Also, there was no evidence of enhancement of the 13.0 kb band in those individuals (both patients and controls) serologically typed as DR4, blank (see Figs 2 and 3), which there might have been if some of these individuals were DR4,4 homozygotes and the 13.0 kb band was associated with DR4 rather than with RA (theoreti-

cally DR4, blank individuals could either be DR4,4 homozygotes or be heterozygous for DR4 and a DR antigen not typed for). The same analysis also demonstrated a negative association between the 7.2 kb *DraI* DQ β RFLP and RA, which also fell short of significance. No correlation was seen between the presence or absence of the 13.0 and 7.2 kb bands. None of the other polymorphic bands showed any significant associations with RA or with the DR4 status of the individuals despite enhancement of the 6.8 kb and 2.4 kb bands in the original RA pool.

Possible correlations of the 13.0 and 7.2 kb *DraI* DQ β associated RFLPs with Dw and DQ types were investigated in eight HLA-DR4 homozygous typing cell lines. Neither band correlated with any of the Dw4, Dw10, Dw13, Dw14, or DKT2 specificities associated with DR4 (Fig. 4). In addition, there was no absolute correlation between the 13.0 kb band and DQ status of the cell lines where known: lines THO, FLE, MAY, and BM14 have been serologically typed as TA10 negative¹⁶—that is, they do not correspond to the DQw7 split of DQw3, whereas they all showed the 13.0 kb RFLP. Line JAH is also TA10 negative, but this lacked the 13.0 kb band. Line JHA is TA10 positive (DQw7) but also lacked the 13.0 kb fragment. None of the other polymorphic bands correlated with Dw or DQ type. There was evidence, however, for a possible diallelic DQ β RFLP system within the DR4 haplotypes of the cell lines, with 13.0 and 2.4 kb bands corresponding to one 'allele' and 7.2 and 3.0 kb bands corresponding to the other.

Finally, HLA-DP associated RFLPs were examined by individually digesting DNA samples from 20 patients with RA and from 20 controls with five restriction enzymes (*TaqI*, *MspI*, *RsaI*, *DraI*, and *PstI*) and hybridising the blotted filters with DP

Fig. 2 *DraI* DQ β associated restriction fragment length polymorphisms (RFLPs) in a sample of patients with rheumatoid arthritis. The DR types of the patients are marked on the digest lanes.

Fig. 3 *DraI* DQ β associated restriction fragment length polymorphisms (RFLPs) in a sample of healthy controls. The DR types of the controls are marked on the digest lanes.

and DP β probes. Although some RFLPs were detected—for example with TaqI and DP β , none proved to be RA associated (data not shown).

Fig. 4 *DraI* DQ β RFLP analysis of eight HLA-DR4 homozygous typing cell lines. The Dw types of the homozygous typing cell lines used are marked on the digest lanes.

Table 2 Prevalence of *DraI* DQ β associated restriction fragment length polymorphisms in patients with rheumatoid arthritis (RA) and in controls. Values are Nos (%)

	Band sizes (kb)					
	13.0	7.2	6.8	3.0	2.7	2.4
Patients with RA	21/26 (81)	17/22 (77)	20/26 (77)	13/22 (59)	14/24 (58)	10/14 (71)
Controls	24/39 (62)	30/32 (94)	21/32 (66)	21/28 (75)	15/28 (54)	22/29 (76)
Probability	0.06	0.07	0.15	0.12	0.21	0.27

Among HLA-DR4 positive patients with RA and controls the 13.0 kb band was present in 14/16 patients (88%) and 17/29 controls (59%); $p=0.04$. Bands were only scored as present or absent when an unambiguous assignment could be made. Probability values were calculated using 2x2 contingency tables and Fisher's exact test.

apparent RFLP associations with RA seen in the DNA pools but not observed in the individual analyses of the expanded panels were due to sampling variation in the relatively small pools of DNA samples from 11 patients and 11 controls. We feel, however, that rapid screening for disease associated RFLPs by the DNA pooling method is still valid as strongly disease associated RFLPs are unlikely to be overlooked, even in the relatively small pools examined in this study. It is true that RFLPs showing only subtle associations with RA may be undetected by this approach, but our aim was to detect HLA class II genetic lesions showing associations with RA stronger than the classic DR4-RA associations, if such lesions do exist. Nevertheless, our results did show a significant positive association between a 13.0 kb DraI DQ β associated RFLP and RA when DR4 positive patients with RA and controls were considered alone. A similar association was seen among all patients with RA and controls, but at a non-significant level. Examination of these results indicates that this RFLP association is with RA, rather than simply a reflection of a primary association with DR4. The same enzyme/probe combination also showed a non-significant negative association between a 7.2 kb band and RA. Thus our data demonstrate a trend towards association between DraI DQ β associated RFLPs and RA.

Various studies have indicated associations between RA and either or both of the Dw4 or Dw14 cellular specificities associated with DR4,¹⁹⁻²¹ while positive DQ β RFLP-TA10 (DQw7)-RA associations have been reported in some other studies.^{5, 22} RFLP analysis of DR4 homozygous typing cell lines showed that the 13.0 kb DraI DQ β associated band did not correlate with any of the Dw4, Dw10, Dw13, Dw14, or DKT2 specificities. Nor was there any absolute correlation with the TA10 serological specificity identifying the DQw7 split of DQw3 (found in DR4 haplotypes). Thus the 13.0 kb RFLP appears to be a novel genetic marker showing a positive association with RA susceptibility.

No RA associated RFLPs were detected with either of the DP probes. This was not unexpected as DP is not in such strong linkage disequilibrium with DR, and DP antigens show few reported associations with RA or other autoimmune diseases, though certain DP antigens may have some protective role in RA.¹⁹

Our results demonstrate an absence of DR β , DQ α , DP α , and DP β RA associated RFLPs, while a sole DQ β RFLP, showing a weak positive association with RA, was detected. Although numbers of patients and controls examined were small, the absence of strongly RA associated RFLPs remains valid and is in broad agreement with the findings of

parallel studies.^{5, 22} Despite the well documented association of DR4 with RA it is noteworthy that no DR β RA associated RFLPs were found. It is of considerable interest that this and other studies on RA point to variation in at least the DQ β gene as conferring disease susceptibility,^{5, 22} but DQ β RFLPs have also been implicated.⁵ In addition, DQ β RFLPs have been detected in coeliac disease,²³ narcolepsy,^{24, 25} pemphigus vulgaris,²⁶ and insulin dependent diabetes mellitus.^{27, 28} Furthermore, in insulin dependent diabetes mellitus direct gene sequencing studies have identified a single amino acid change in the DQ β chain of all haplotypes associated with this diabetes.²⁹ Similar findings have also been obtained for DQ β and pemphigus vulgaris, while DR β sequence variation has been detected in RA associated haplotypes (McDevitt, unpublished results). It may well be that in RA, as in other autoimmune diseases, DQ β allelic polymorphisms determine, at least partly, the specificity and extent of the disease associated autoimmune response through T cell help or suppression, or both.

The paucity of strongly RA associated HLA class II RFLPs shown by this study indicates that the class II genetic element in RA, a heterogeneous and almost certainly polygenic disease, may well be weak and of the same order of magnitude as that indicated by classical HLA-DR serological analysis. Nevertheless, the results presented in this paper implicate DQ β rather than DR β sequence variation in conferring some susceptibility to RA.

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