Evidence for negative association of the chemokine receptor CCR5 d32 polymorphism with rheumatoid arthritis

V Pokorny, F McQueen, S Yeoman, M Merriman, A Merriman, A Harrison, J Highton, L McLean

Background: Ligands of chemokine receptor CCR5, including MIP-1α, MIP-1β, and RANTES, have been implicated in rheumatoid arthritis.

Objective: To test whether CCR5 d32 polymorphism has a negative association with rheumatoid arthritis in a New Zealand cohort.

Methods: 516 white patients with rheumatoid arthritis and 985 healthy controls were investigated by PCR amplification of the region flanking the known CCR5 d32 deletion, and the frequencies of CCR5 d32 compared. An early rheumatoid arthritis (ERA) cohort of 92 patients was followed prospectively for two years; disease severity and outcome were correlated with CCR5 d32 status.

Results: 12 control subjects (1.2%) were homozygous for d32; no d32 homozygous rheumatoid patients were detected (p = 0.012); 56 patients (10.9%) were heterozygous for the d32 polymorphism (d32/wt), compared with 169 controls (17.2%) (p = 0.0011). The CCR5 d32 allele frequency was lower in the rheumatoid patients than in the controls (frequencies of 0.054 and 0.098, respectively; p = 3.7 × 10⁻⁵). The frequency of CCR5 d32 did not differ significantly according to disease severity or outcome in the prospective ERA cohort, nor with HLA-DRB1 status.

Conclusions: This study provides further evidence for a protective effect of the CCR5 d32 variant on rheumatoid arthritis, consistent with a role for CCR5 and its ligands in disease pathogenesis.

METHODS

Study subjects
We recruited 516 unrelated white New Zealanders with rheumatoid arthritis (368 female, 148 male) who fulfilled the revised criteria of the American Rheumatism Association (later the American College of Rheumatology (ACR)) for the diagnosis of rheumatoid arthritis. They were patients attending rheumatology outpatient clinics at tertiary referral centres within New Zealand. Ninety two rheumatoid patients were part of a prospective early rheumatoid arthritis (ERA) study and have had regular detailed follow up as reported previously.13 Healthy control subjects comprised 985 white donors (526 female, 459 male) attending the Auckland Regional Blood Service. The study had ethics committee approval and all subjects provided written informed consent.

Genotyping and analysis
Genomic DNA was extracted from anticoagulated whole blood using a sucrose lysis buffer and DNAzol (Invitrogen), and then amplified by polymerase chain reaction (PCR) using oligonucleotide primers spanning the CCR5 d32 region (cDNA nucleotides (nt) 794–825 relative to GenBank NM000579): CCR5 P1 5′–TTT ACC AGA TCT CAA AAA GAA G (sense) and CCR5 P2 5′–GGA GAA GGA CAA TGT TGT AGG (antisense). Reaction mixtures (15 µl) containing DNA (100 ng), oligonucleotide primers (0.5 µM), dNTPs (200 mM), buffer consisting of 10 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.25 mM MgCl₂; and 1 unit of DNA polymerase were heated to 94°C for three minutes and then subjected to 32 cycles of amplification with denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds.

Abbreviations: ERA, early rheumatoid arthritis; RANTES, regulated upon activation, normal T cell expressed and secreted; SE, shared epitope
35 amplification cycles of 94°C for 30 seconds, 62°C for one minute, and 72°C for one minute, followed by a final elongation cycle of 72°C for five minutes. The PCR products were visualised on ultraviolet transilluminated, ethidium bromide stained 2% agarose gels after electrophoresis, yielding PCR products of 274 and 242 bp for the CCR5 wt and d32 alleles respectively (fig 1). Typing for HLA-DRB1 was done with a sequence specific primer PCR or a single stranded oligoprobe PCR (DRB1 LiPA, Innogenetics). High resolution HLA-DRB1 typing was done on ERA subjects by DNA sequencing, and the HLA-DR shared epitope (SE) status was considered positive in the presence of HLA-DRB1*0101, *0401, *0404, *0405, *0408, *1001, or *1402; no other DRB1 alleles with the codon 70–74 QKRAA, QRRAA or RRRAA sequence were detected.

Subjects were considered to be homozygous if the low resolution typing indicated only one HLA-DRB1 type and manual review of the sequencing electropherograms showed a single peak for every nucleotide.

**Statistics**

The two tailed Fisher exact test was carried out to determine the statistical significance of differences in genotype and allele distributions, and the Mann–Whitney U test was used to compare continuous variables. Prism version 3.00 and InStat version 3.00 (GraphPad Software) were used for statistical analysis.

**RESULTS**

The genotype and allele frequencies of the 32 bp deletion are summarised in table 1. Eleven per cent of patients were heterozygous for the d32 polymorphism (d32/wt), compared with 17% of controls (p = 0.0011). In the rheumatoid patients the CCR5 d32 allele frequency (0.054) was significantly lower than in the healthy controls (0.098; odds ratio = 0.51 (95% confidence interval (CI), 0.37 to 0.69); p = 3.7 \times 10^{-5}). Although 12 d32/d32 deletion homozygotes were present in the 985 controls (1.2%), none was detected among the 516 rheumatoid patients (p = 0.012). In the ERA cohort, after correcting for multiple testing, CCR5 d32 status was not associated with a significant difference in disease severity as assessed by baseline or follow up tender or swollen joint counts, rheumatoid factor status, erythrocyte sedimentation rate, C reactive protein, disease modifying antirheumatic drug usage, health assessment questionnaire/disease index, radiographic erosion status, or (in a subcohort described

<table>
<thead>
<tr>
<th>Table 1</th>
<th>CCR5 genotype and allele frequencies</th>
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<tr>
<td>(a) Genotype frequencies</td>
<td></td>
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<tr>
<td>Population</td>
<td>Subjects</td>
</tr>
<tr>
<td>RA</td>
<td>516</td>
</tr>
<tr>
<td>Controls</td>
<td>985</td>
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<tr>
<td>(b) Allele frequencies</td>
<td></td>
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<tr>
<td>Population</td>
<td>Chromosomes</td>
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<tr>
<td>RA</td>
<td>1032</td>
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<tr>
<td>Controls</td>
<td>1970</td>
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</table>

Values are the number (frequencies (freq)) of subjects (a) and chromosomes (b).

*p=0.0011; **p=0.012; ***p=3.7 \times 10^{-5}, patients v controls.

RA, rheumatoid arthritis.
previously by dominant wrist magnetic resonance image scoring (data not shown). In the ERA group, 13 (14.1%) were heterozygous (d32/wt). None was d32/d32 homozygous.

The CCR5 d32 genotypes complied with the Hardy-Weinberg equilibrium, and the frequencies in the control subjects were compatible with those in other published studies of European white subjects.\(^1^\) - 3 Low resolution HLA-DRB1 typing had been done for 475 of the rheumatoid patients and 912 controls. As expected, carriage of a DRB1*04 allele was significantly higher in rheumatoid (276/475, 53.4%) than in control subjects (199/912, 21.2%) (\(p<0.0001\)). Among the 92 ERA patients, 48 (52.2%) were DR4 positive and 67 (72.8%) were SE positive. The corresponding HLA-DRB1*04 and SE positivity frequencies did not differ significantly according to CCR5 d32 status (data not shown).

**DISCUSSION**

We investigated the CCR5 gene 32 bp deletion polymorphism in a large cohort of rheumatoid patients and controls, comprising over 1500 individuals. In this rheumatoid arthritis cohort the CCR5 d32 allele frequency in patients (5.4%) was significantly reduced in comparison with that in the healthy individuals (9.8%), and there was a striking absence of rheumatoid patients who were homozygous for the mutated receptor gene. We did not show an effect on disease severity, nor any interaction of the CCR5 d32 polymorphism with HLA-DRB1*04 or SE status; however, our cohort size may have been too small to show these effects.

This study is the first to demonstrate a significant negative association of the CCR5 d32 polymorphism with rheumatoid arthritis \((p<0.0001)\). We replicated the finding of Gomez-Reino et al., who also reported a negative association of the CCR5 d32/d32 homozygous genotype with rheumatoid arthritis (no d32/d32 individuals among 673 Spanish patients with rheumatoid arthritis). Three other studies examining the association of CCR5 d32 with rheumatoid arthritis have reported no nominally significant differences \((p>0.05)\) in frequency between rheumatoid cases and healthy controls \((d32/wt)\) genotype frequencies 19.2% and 13.7%; 24.5% and 18.4%; and 16.8% and 11.3%, respectively).\(^8^\) - 11 This apparent inconsistency may reflect one or a combination of several factors: heterogeneity in the detection and referral patterns of rheumatoid arthritis according to disease severity; differences in rheumatoid arthritis aetiology between the ethnic and geographic origins of subjects; or inadequate power. Some studies of white subjects of British, Danish, and Spanish origin have provided no evidence for an association of CCR5 d32 with rheumatoid arthritis per se,\(^8^\) - 11 whereas others, such as the present study, in white New Zealanders, and that of Gomez-Reino et al.\(^8^\) in white Spanish subjects, do provide evidence for an association. Thus there is no indication that the ethnic origin of the subjects is a major factor in the discrepancy in CCR5 d32 association data between studies.

We speculate that inadequate power of studies is more likely to account for the lack of replication. The wild type allele at CCR5 d32 confers an odds ratio of 1.89 (95% CI, 1.39 to 2.57) for developing rheumatoid arthritis in our cohort. Using a conservative odds ratio estimate of 1.39 (the lower bound of the confidence interval), the largest of the three studies to find no evidence for an association (Cooke et al., 278 patients) had only 16% power \((\alpha = 0.01)\). An equivalent post priori calculation reveals that our cohort had 44% and that of Gomez-Reino et al.\(^8^\) 34% power \((\alpha = 0.01)\). (Power calculations were made as previously described).\(^8^\) Although these power calculations are stringent (using a conservative odds ratio estimate and \(\alpha = 0.01\)), they do indicate that, even with over 500 cases, association studies are still underpowered, and a degree of luck is required to detect an effect of CCR5 d32. To have 80% power \((\alpha = 0.01)\) will require at least 1400 cases and an equal number of controls. Stratifying such underpowered cohorts in an attempt to reduce phenotype heterogeneity (disease severity, for example) will further reduce power.

Two other studies done on different populations each identified two rheumatoid patients with a homozygous CCR5 d32/d32 genotype, suggesting that CCR5 deficiency does not completely prevent rheumatoid arthritis. Furthermore, in the study of John and colleagues, four patients with inflammatory polyarthritis were homozygous for d32/d32. However, only two of these patients met ACR criteria for rheumatoid arthritis and both of these subjects remained rheumatoid factor negative and non-erosive five years after onset. Overall, the findings are consistent with CCR5 ‘‘deficiency’’ conferring a degree of protection against this disease. Indeed, CCR5 d32 has been associated with protection against various other immunological disorders including type 1 diabetes, asthma, multiple sclerosis, glomerulonephritis, allograft rejection, and HIV progression.

Individuals homozygous for the 32 bp deletion allele lack the functional membrane receptor, while heterozygous d32 carriers have reduced expression. The reduced level of functional chemokine receptors on the cell surface could impair the influx and activation of inflammatory cells. The involvement of inflammatory pathways using CCR5 in the pathogenesis of rheumatoid arthritis suggests the possible utility of pharmacological blockade of CCR5 or its ligands.

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Conflict of interest: LM is a former employee of AstraZeneca and current employee of Merck and Co.; the work described in this paper was conducted before taking up these appointments.

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