CC Chemokine Receptor Gene Polymorphisms in Czech Patients with Pulmonary Sarcoidosis

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Genes for the chemokine receptors CCR5 and CCR2 are characterized by polymorphisms resulting in a nonfunctional receptor expression. Ligands for CCR2 and CCR5 (chemokines monocyte chemotactic protein-1 [MCP-1] and RANTES) are implicated in the pathogenesis of sarcoidosis. We have, therefore, analyzed polymorphisms of CCR5 (32-bp deletion in CCR5 gene [Δ32]) and of CCR2 (replacement of valine by isoleucine in CCR2 gene [64I]) in 66 Czech patients with sarcoidosis in comparison with a representative sample of Czech normal population. The frequencies of CCR5Δ32 and CCR2-64I polymorphisms in patients with sarcoidosis were different from that in control subjects. CCR5Δ32 allelic frequency was significantly increased in patients. By contrast, the CCR2-64I allele was more frequent in control subjects; however, the difference did not attain significance. Interestingly, the CCR5Δ32 allele was associated with clinically more apparent disease: it was present in 39.1% of patients requiring corticosteroids but only in 16.7% patients who did not need therapeutic intervention (odds ratio [OR] = 2.9). When patients requiring corticosteroids were compared with control subjects, the differences in the CCR5Δ32 frequencies were enhanced ($p < 0.01$). In conclusion, the observed association of CCR5Δ32 and CCR2-64I with sarcoidosis implicates a role for these polymorphisms in disease susceptibility and protection.

Sarcoidosis is a multisystem disorder of unknown origin most frequently affecting the lungs, where it typically appears as $CD4^+$ T-lymphocyte and macrophage alveolitis (1). Interplay between chemotactic cytokines (chemokines), and their receptors is considered to be crucial for transmigration of lymphocytes and monocytes from the circulation to the bronchoalveolar space (2). CC chemokines RANTES (regulated upon activation, normal T-cell expressed and secreted; CCL5), monocyte chemotactic protein-1 (MCP-1; CCL2), and macrophage inflammatory protein-1α (MIP-1α; CCL3) have been implicated in this process (3–6).

Two of the receptor molecules, which may bind the aforementioned chemokines, namely CC chemokine receptor (CCR) 5 and CCR2, are characterized by gene polymorphisms. A 32-bp deletion in the CCR5 gene (CCR5Δ32) results in a nonfunctional surface receptor molecule unable to bind its chemokine ligands RANTES, MIP-1α, and MIP-1β (7). A substitution mutation (replacement of valine by isoleucine in the transmembrane region) has been described in the CCR2 gene (CCR2-64I) (8); CCR2 is a receptor for MCP-1 and also its homologues MCP-2 to MCP-5 (7). Both CCR mutations have been implicated in the pathogenesis of human immunodeficiency virus (HIV) infection, and the presence of the mutated allele or alleles confers varying degree of anti-HIV protection that is reflected in slower disease progression (9).

To investigate if these polymorphisms in "candidate" chemokine receptor genes are relevant for the development of alveolitis in sarcoidosis, we have investigated the distribution of the wild-type and mutant alleles of CCR2 and CCR5 in Czech patients with sarcoidosis in comparison with healthy control subjects. Further, to explore if these polymorphisms may affect the clinical course of the disease, we have analyzed the association of particular genotypes with two groups of patients: those requiring corticosteroids for control of disease activity and those who do not need corticosteroids.

METHODS

Study Population

Chemokine receptor polymorphisms were determined in 66 patients with sarcoidosis and in 386 control subjects (Table 1). The control group consisted of unrelated healthy subjects, participants of the Czech Bone Marrow Donor Registry (CNRDD), who agreed to the anonymous usage of their DNA for research purposes. Absence of lung disease in the control subjects was checked during their registration for the Registry by health questionnaire and interview. In patients, the diagnosis of sarcoidosis was based on typical clinical features together with granulomas on histopathologic examination. The diagnosis was further supported by a lymphocytic, $CD4^+$ bronchoalveolar lavage. The blood for DNA extraction was obtained at the first day of presentation and after a period of 2 yr, the course of the disease was reviewed.

The patients were subdivided according to the need for corticosteroid treatment into two groups: patients who received treatment (n = 46) and patients in whom treatment was not necessary, i.e., the disease resolved spontaneously (n = 18); in two patients data on therapy were unavailable. The treatment scheme did not differ from that recommended in the International Statement on Sarcoidosis (10): Treatment with steroids was indicated according to an accepted protocol: (*1*) all patients with chest X-ray Stage III disease at presentation; (*2*) patients with progressing and/or symptomatic Stage II disease; and (*3*) patients with persistent Stage I or II disease. Patients with persistent disease were treated after at least 6 mo of disease observation.

The study was performed with the approval of the ethics committee of the Medical Faculty and University Hospital Olomouc.

CCR Genotyping and Statistical Analysis

Assessment of chemokine receptor polymorphism. DNA was extracted using a standard salting out procedure. CCR5 and CCR2 wild-type and mutant alleles were typed by polymerase chain reaction using sequence-specific primers (PCR-SSP); for primer sequences, *see* Table 2. For characterization of the CCR2 polymorphism, two amplification reactions were used: the first with primers specific for the wild-type allele sequence, the second with primers specific for the sequence of the mutant allele. In the case of a homozygous wild-type individual, the

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TABLE 1

CHARACTERISTICS OF THE INDIVIDUALS GENOTYPED FOR CCR2 AND CCR5

	Patients with Sarcoidosis	Control Population (CCR2)	Control Population (CCR5*)
n	66	80	386
Male:Female ratio	29:37	47:33	250:136
Age, mean ± SD	46.5 ± 11.7	34.1 ± 8.8	33.6 ± 8.3
Age, range	25–78	19–69	13–64

* This control population has been previously used in our study of CCR5Δ32 frequency in the normal Czech population (Reference 12).

product was observed only in the first reaction; in the case of a homozygous mutant individual the product was detected only in the second reaction. When the typed proband was heterozygous (CCR2, CCR2-64I), the products were detected in both the CCR2-specific and CCR2-64I-specific reactions. The composition of PCR reaction-mix, cycling protocol, and electrophoresis conditions was adopted from "phototyping" methodology (11). The CCR5 polymorphism was characterized as previously described (12) by one amplification reaction with primers flanking the region containing the 32-bp deletion. The wild-type allele was detected as a 182-bp fragment; the CCR5Δ32 allele was detected as a 150-bp fragment. In a heterozygous individual, both fragments were detected.

Statistics. Comparisons were made between allelic (gene) and phenotype frequencies in the disease and control populations; the term "phenotype frequency" (i.e., carriage rate) gives the number of individuals carrying one (or two) copies of a particular allele on one or both (maternal and paternal) chromosomes. Comparisons were also made between patients with different clinical courses (patients requiring treatment or patients with spontaneous resolution of the disease without treatment). The data sets were compared using a standard 2×2 chi-square analysis by SIGTEST, a computer-based program that uses a Woolf-Haldane correction in cases of small numbers (13). This program has a facility for the calculation of chi-square statistics, the significance value, and the relative risk (odds ratio [OR]). The control populations were tested for conformity to the Hardy-Weinberg equilibrium using a 2×2 chi-square test between observed and expected numbers.

RESULTS

The Czech (control and patient) populations were found to be in Hardy-Weinberg equilibrium with regard to the distribution of CCR2 and CCR5 genotypes ($p > 0.05$). Phenotype and gene frequencies for CCR2 and CCR5 polymorphisms are summarized in Table 3. The frequencies of CCR2-64I in patients were different from control subjects (*see* Table 3) although these differences did not attain statistical significance. By contrast, the allelic and phenotype frequency of CCR5Δ32 was significantly higher in patients than in control subjects ($p = 0.02$ and $p = 0.03$, respectively). The frequency of homozygous mutant (CCR5Δ32) individuals was 1.5% (1/66) in the patient group and 0.3% in the control group (1/386); no homozygotes with CCR2-64I were detected in either control individuals or patients.

When the patients were divided according to the necessity for treatment, more pronounced differences in CCR5Δ32 frequency emerged. In the group of 46 patients requiring treatment there were 18 (39.1%) CCR5Δ32-positive individuals by comparison with three of 18 patients (16.7%) with spontaneous disease resolution (OR = 2.9; $p = 0.1$); phenotype frequency of CCR5Δ32 in the normal population is 21.2% (Figure 1).

Importantly, when only the patients requiring treatment were included in the analysis (based on the fact that they have more pronounced disease), the differences between the allelic and phenotype frequencies of CCR5Δ32 in the normal population and patients with sarcoidosis became highly significant (allelic frequency, $p = 0.005$; phenotype frequency, $p = 0.007$). Also, the relative risk of sarcoidosis for the subjects with CCR5Δ32 allele increased from 1.9 to 2.4 (95% confidence interval [CI]: 1.3 to 4.5).

TABLE 3

CCR2-64I AND CCR5Δ32 FREQUENCIES IN PATIENTS WITH SARCOIDOSIS AND CONTROL SUBJECTS

CCR2-64I	Controls (*n = 80*)	Patients (*n = 65**)	p Value†
Allelic (gene)	11.9%	6.9%	0.17
Antigen (phenotype)	23.8%	13.8%	0.14‡
CCR5Δ32	(*n = 386*)	(*n = 66*)	p Value
Allelic (gene)	10.8%	17.4%	0.02
Antigen (phenotype)	21.2%	33.3%	0.03§

* In one patient, CCR2 polymorphism could not be defined.
† Patients compared with control subjects.
‡ Odds ratio (OR) CCR2-64I 0.5 (95% CI: 0.2–1.3).
§ OR CCR5Δ32 1.9 (95% CI: 1.1–3.2).

DISCUSSION

This study has shown an association between chemokine receptor polymorphisms and pulmonary sarcoidosis. Primary

TABLE 2

SEQUENCES OF PRIMERS USED FOR PCR-SSP DETECTION OF CCR2 AND CCR5 POLYMORPHISMS*

Code	Gene	Sequence
440	CCR2	5′ g T g g g C A A C A T g C T g g T C A
441	CCR2	5′ C C C A A A g A C C C A C T C A T T T g
442	CCR2	5′ g T g g g C A A C A T g C T g g T C g
220	CCR5	5′ C T T C A T T A C A C C T g C A g C T C T
221	CCR5	5′ C A C A g C C C T g T g C C T C T T C T T C

* CCR2 wild-type allele was typed using primers 441 + 442, CCR2-64I allele using primers 441 + 440. CCR5 wild-type and mutant alleles were typed with primers 220 + 221.

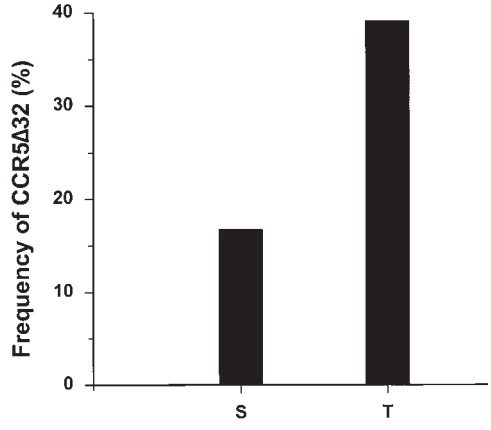

Figure 1. CCR5Δ32 phenotype frequencies in patients with sarcoidosis with different disease course. S = patients in whom disease resolved spontaneously (n = 18); T = patients who required steroid treatment (n = 46). Treated patients (T) compared with untreated patients (S), p = 0. 1. OR = 2.9 (95% CI: 0.8–10.5). The phenotype frequency of CCR-5Δ32 in normal Czech population is 21.2%.

analysis (comparison of sarcoidosis as a whole with the controls) has shown a decrease of the CCR2-64I allele, and a significant increase in the frequency of the CCRΔ32 allele. Disease subset analysis of those patients requiring therapy compared with those with spontaneous resolution of sarcoidosis showed that the presence of the mutant CCR5Δ32 allele was confined to the more pronounced clinical course of the disease. Subjects with the mutant CCR5 allele were three times more prone to require treatment than those without the allele, i.e., the presence of the mutant CCR5 allele carried an increased relative risk (OR = 2.9) of the need for treatment. Furthermore, if the cases with a more benign clinical course (those not requiring treatment) were excluded from the analysis, the difference between CCR5Δ32 allelic and phenotype frequencies in patients with sarcoidosis and control subjects became highly significant.

The results are therefore consistent with the hypothesis that polymorphisms in CCR2 and CCR5 receptors for the chemokines MCP-1 and RANTES (themselves implicated in the pathogenesis of sarcoidosis) may affect the development of this disease. In this regard, our patient population was representative of sarcoidosis with respect to the male:female ratio, age range, and clinical course of the disease. Second, because of different frequencies of CCR5Δ32 allele around the world (14) only unrelated Czech patients and unrelated Czech control subjects who were white were included in the study to ensure that it is not confounded by ethnic mixing. Third, technical problems in our analysis or data generation are unlikely as the allele and genotype frequencies of both polymorphisms do not deviate from what would be expected to occur randomly in the population according to Hardy-Weinberg equilibrium (15). Further, the adequacy of the genotyping approach is evidenced by the agreement between the published CCR2-64I (16) and CCR5Δ32 (14) frequencies and our results.

Our study is not the first investigation of chemokine receptors and their polymorphisms in sarcoidosis. Agostini and coworkers (17) found increased cell surface expression of CXCR3 (receptor for chemokines such as gamma interferon inducible protein-10 [IP-10]) on T cells from sarcoid lung. Recently, Hizawa and coworkers (18) have reported a significantly decreased frequency of the CCR2-64I allele in Japanese patients with sarcoidosis. Our results agree with those obtained in the Japanese population (18) and imply that a possible protective effect of the CCR2-64I polymorphism is present also in the white population, though less markedly. There have, however, been no reports to date concerning CCR5 expression or CCR5 gene polymorphisms in sarcoidosis. We can, therefore only speculate about the mechanism by which these CC chemokine receptor polymorphisms differentially contribute to susceptibility to sarcoidosis.

In sarcoidosis, an oligoclonal T helper cell, type 1 (Th1) T-cell-mediated immune response elicited by unknown antigen leads to an accumulation of activated Th1 T cells at sites of inflammation and contributes to the development of delayed type hypersensitivity (DTH) granulomas (1, 2, 19). Interestingly, T-cell lines from a CCR5-deficient individual (CCR5Δ32 homozygote) contained high numbers of Th1 cytokine (interferon gamma [IFN-γ], interleukin-2 [IL-2]) positive cells (20), and also T cells of the CCR5-deficient mice showed increased production of IFN-γ and had an enhanced DTH reaction with increased $CD4^+$ infiltration (21). By contrast, in CCR2-deficient mice an impaired DTH response and decreased production of Th1-type cytokines was observed (22). These findings allow the speculation that in individuals carrying the CCR5Δ32 allele, there is an increased predisposition to a Th1-type immune response and subsequent granuloma formation, whereas in the individuals with CCR2-64I Th1 reactivity is attenuated. This interpretation may be challenged by the recent findings of impaired Th2 cytokine–mediated lung granulomas elicited by schistosomal antigen in CCR2-deficient mice (23). However, according to Kunkel and coworkers (24), a switch to Th2-type T cells may occur in patients with sarcoidosis evolving toward lung fibrosis. On the other hand, our hypothesis, that CCR5Δ32 polymorphism favors a Th1-type immune response is supported by the observation of a decreased frequency of CCR5 deletion mutation in asthma, a typical Th2 disease (25).

The presence of a 32-bp deletion in the CCR5 loci on both chromosomes results in a nonfunctional surface receptor molecule unable to bind its chemokine ligands RANTES, MIP-1α and MIP-1β (7). All but one of our CCRΔ32-positive patients was heterozygote. It is known that heterozygosity in CCR5Δ32 results in a 50% decrease of CCR5 molecule expression on the cell surface (26), and, therefore, we have to ask whether the deletion mutation and its gene dosage have a real impact on cell recruitment. In the situation of partially or fully nonfunctional CCR5, monocytes and T cells could migrate to the sarcoid lung using alternative pathways. For example, the chemokine RANTES, a CCR5 ligand implicated in lymphocyte migration in sarcoidosis (3, 4), may exert its chemotactic effects by compensatory binding to other receptors such as CCR1 or CCR3, or both. Alternatively, cellular influx into sarcoid alveoli may be encouraged by other chemokines such as IP-10, which binds to the CXCR3 receptor (17). CXCR3, together with CCR5, characterizes cell subsets associated with Th1-type inflammatory reactions (27, 28) and importantly, cellular expression of CXCR3 is upregulated in sarcoid patients (17).

The existence of compensatory mechanisms of cell migration suggests that the pathogenetic role of the CCR5Δ32 in sarcoidosis may not be related to impaired cell migration. Alternatively, a stronger and prolonged immune response may develop in CCR5Δ32-positive individuals with sarcoidosis as a result of altered signaling through CCR5. This concept is supported by the results of gene targeting studies, which have recently indicated a novel role for CCR5 in modulating T-cell function. Signaling through CCR5 might function as part of a negative regulatory cycle of T-cell activation (21) and it was suggested that there might be a cross-talk between T-cell receptor and chemokine signaling pathways (29).

CCR2 and CCR5 genes are (together with CCR1 and 3) located on chromosome 3p21.3, within a gene cluster of 350 kb (30). Genome-wide screening has implicated the CCR5 region, defined by the marker DS3S1573, in the susceptibility to Crohn's disease (31), which shares some pathogenetic features (Th1 granulomatous inflammation and alveolitis) with sarcoidosis and may occur within families (32, 33). Therefore, another explanation of the observed association of CCR5 and CCR2 polymorphisms with sarcoidosis is, as already suggested by Hizawa and coworkers (18), the possibility of the existence of linkage disequilibrium with other as yet unknown genes in the CCR cluster. The candidates include CCR5 promoter alleles, some of which have been recently associated with HIV-1 disease progression (34). Alternatively, the observed relationship between presence of CCR5Δ32 and the need for corticosteroid treatment suggests that CCR5 may control the expression of other receptors with pharmacologic function.

In conclusion, we have shown that Czech patients with sarcoidosis have profiles of CCR2 and CCR5 polymorphisms distinct from the normal population; that these chemokine receptor polymorphisms play an as yet undefined role in the development of sarcoidosis; and that CCR5Δ32 is related to clinically more apparent disease. CC chemokine receptor polymorphisms should, therefore, be added to the current spectrum of immunogenetic

factors known to be involved in the pathogenesis of this multifactorial disease. Further work is required to characterize the functional relevance of these polymorphisms.

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