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PREDOMINANCE OF MONONUCLEAR CELLS EXPRESSING THE CHEMOKINE RECEPTOR CCR5 IN SYNOVIAL EFFUSIONS OF PATIENTS WITH DIFFERENT FORMS OF ARTHRITIS

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Objective. **To study the role of the chemokine receptors CCR5 and CCR2 in patients with arthritis.**

Methods. **CCR5 expression on peripheral blood leukocytes was compared with the expression on leukocytes isolated from the synovial fluid of 20 patients with different rheumatic joint diseases. Three additional samples were studied for CCR2 expression. The expression of chemokine receptors on blood and synovial fluid leukocytes was determined by 3-color flow cytometry analysis. To test CCR5 receptor down-modulation from the cell surface, leukocytes were incubated in vitro with a RANTES (regulated on activation, normal T cell expressed and secreted) derivative, aminooxypentane (AOP)–RANTES. Patients were genotyped for the Δ32 CCR5 deletion by polymerase chain reaction.**

Results. **A high percentage of CCR5-expressing CD4+ and CD8+ T cells (74% and 81%, respectively), monocytes (51%), and natural killer cells (35%) was found in the synovial fluid of all patients, whereas in the peripheral blood, only a small percentage of these cells expressed CCR5 (13%, 32%, 7.8%, and 4%, respectively). Infiltration of CCR5-positive leukocytes was not reduced in CCR5-heterozygous patients. A similar, but less pronounced, distribution was observed for CCR2-positive T cells. In vitro, CCR5 was completely down-modulated on synovial fluid leukocytes by AOP-RANTES.**

Conclusion. **The predominance of CCR5-positive mononuclear cells in the synovial effusions of patients with arthritis suggests an important role for CCR5 in the process of joint inflammation, and identifies CCR5 as a possible new target for therapeutic intervention.**

Chemokine receptors are a family of G-protein–coupled proteins with 7 transmembrane helices, and these receptors are expressed on different subgroups of leukocytes. Chemokines, together with adhesion molecules, play an important role in the homing of naive lymphocytes into lymph nodes (1–3) and their subsequent migration into inflamed tissue. The extravasation of leukocytes is thought to be a multistep process that is initiated by a loose interaction between selectins and carbohydrate groups, which results in rolling of leukocytes on the endothelium. Chemokines then induce a tight binding between integrins and adhesion molecules on the endothelium, leading to transendothelial migration (4,5). Within the tissue, leukocyte migration is thought to follow chemokine gradients, which direct the cells toward the site of injury.

In vitro, there is a high degree of redundancy in chemokine–receptor interactions, since many chemokines bind to more than one receptor and vice versa (6). For example, macrophage inhibitory protein 1α (MIP-1α), MIP-1β, and RANTES (regulated on activation, normal T cell expressed and secreted) bind to CCR5, but RANTES also binds to CCR1 (7) and CCR3 (8). To determine whether there is a higher selectivity for the attraction of CCR5-expressing leukocytes in vivo, we compared the CCR5 expression on leukocytes from the peripheral blood and that on leukocytes isolated from the synovial effusions of patients with various forms of arthritis.

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In the peripheral blood, the chemokine receptor CCR5 is expressed on a subset of CD4+ and CD8+ T cells, as well as on a small percentage of monocytes and natural killer (NK) cells (9). Approximately 20% of the CD4+ and 30% of the CD8+ T cells express CCR5 and belong mainly to the memory T cell subset. Furthermore, ~20% of whites are heterozygous for an internal 32-basepair deletion in the CCR5 allele, leading to a reduced surface expression of this receptor (10–12).

In the present study, we also studied the expression of the chemokine receptor CCR2, in 3 additional patients. CCR2 is the receptor for monocyte chemotactic proteins (MCP-1–MCP-5) and is expressed on a subset of CD4+ and CD8+ T cells, as well as on monocytes and other lymphocyte subpopulations. Of interest, MCP-2 is a common ligand for CCR2 and CCR5 (13).

Various cytokines and chemokines have been detected in the synovial fluid and synovial tissue of patients with rheumatic diseases (14,15). It is thought that tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), which are mainly secreted by infiltrating macrophages, contribute to pannus formation and cartilage destruction in rheumatoid arthritis (RA). In the first clinical studies, the neutralization of TNF in patients with severe RA resulted in reduced disease activity (15,16). Moreover, a variety of CXC and CC chemokines have been found in the synovial fluid and synovial tissue of RA patients (17–20). Among these, the CC chemokines RANTES, MIP-1α, and MIP-1β might be responsible for the preferential attraction of T cells and monocytes that express the CCR5 chemokine receptor. Cell infiltrate in the synovial tissue of RA patients consists mostly of macrophages, T lymphocytes, and plasma cells and, to a lesser extent, of dendritic cells and activated fibroblasts (21,22). CCR5- and CXCR3-positive lymphocytes have been found in the inflammatory infiltrates of patients with RA, inflammatory bowel disease, and chronic vaginitis (23,24).

It is assumed that T helper cells with a Th1 phenotype, characterized by the production of interferon-γ and IL-2, are of importance in the pathogenesis of RA (25–28). An enrichment of Th1 cells has also been seen in patients with Lyme arthritis (29). There is growing evidence that the pattern of chemokine receptor expression on lymphocytes is related to their state of activation (30) and the type of inflammatory response. T helper cells expressing CCR3 and CCR4 were found to belong to the Th2 subset (31,32), which is involved in allergic reactions and immunity to parasites. In contrast, T helper cells displaying a Th1 phenotype were shown to preferentially express CCR5 and CXCR3 (31,33,34).

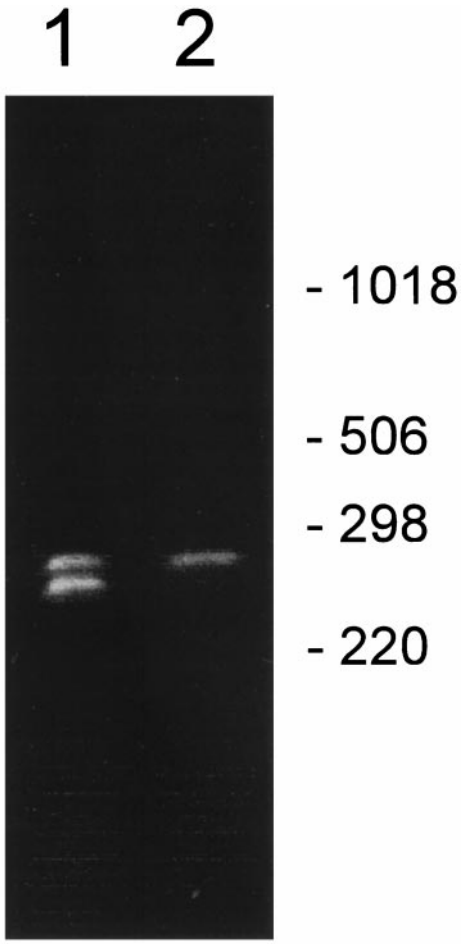

Figure 1. Analysis of the CCR5 genotype by polymerase chain reaction, as described in Patients and Methods. Lane 1, Heterozygous genotype; lane 2, wild-type genotype. DNA size (in bp) is indicated on the right.

In this study, we analyzed the CCR5 expression on leukocytes isolated from the synovial fluid of 20 patients with different rheumatic joint diseases. In parallel, CCR5 expression and genotype were determined on the peripheral blood leukocytes from the same patients. We found a clear predominance of CCR5-expressing mononuclear cells in the synovial fluid of all patients. The predominance of these cells in the effusion may be due to CCR5-selective immigration or up-regulation of CCR5 expression on cells already present in the effusion.

PATIENTS AND METHODS

Patients. Synovial fluid and peripheral blood samples were simultaneously obtained from patients with gonarthritis

Table 1. Clinical data on 20 patients with gonarthritis analyzed for CCR5 expression on leukocyte subsets*

Patient/sex/age	CCR5 genotype†	Leukocytes/μl	Percentage of PMN/Ly/Mc‡	Disease duration	Diagnosis
1/F/59	+/+	15,200	75/4/21	2 months	RA
2/F/61	+/−	12,000	33/59/8	15 years	RA
3/F/40	+/+	8,800	83/11/6	2 years	RA
4/M/39	+/+	5,500	69/29/2	3 years	RA
5/F/28	+/−	8,000	68/23/8	12 years	RA
6/F/55	+/−	5,500	ND	1 month	RA
7/F/36	+/+	12,900	83/11/6	10 years	AS
8/M/48	+/−	8,400	81/18/1	1 month	AS
9/M/45	+/−	6,100	28/65/7	18 months	AS
10/M/33	+/+	5,200	90/4/6	20 months	AS
11/M/17	+/−	4,600	13/22/65	4 years	PsA
12/F/28	+/+	10,900	94/5/1	3 years	PsA
13/M/51	+/+	11,900	73/23/4	2 years	PsA
14/F/56	+/+	5,000	59/16/25	2.5 years	UcA
15/F/52	+/+	8,600	19/75/6	6 months	UcA
16/F/26	+/+	12,000	81/15/3	12 years	UcA
17/M/42	+/+	3,800	71/22/7	3 months	ReA
18/F/50	+/+	29,300	86/13/1	6 months	Lyme
19/M/44	+/+	48,000	96/2/2	2 days	Gout
20/M/47	+/+	4,900	65/25/10	5 weeks	Gout

* RA = rheumatoid arthritis; ND = not done; AS = ankylosing spondylitis; PsA = psoriatic arthritis; UcA = unclassified arthritis; ReA = reactive arthritis.
† + indicates the normal allele and − indicates the Δ32 mutation.
‡ Leukocytes were differentiated into polymorphonuclear neutrophilic granulocytes (PMN), lymphocytes (Ly), and monocytes (Mc).

who were undergoing diagnostic arthrocentesis. Clinical diagnoses included RA, psoriatic arthritis, ankylosing spondylitis, reactive arthritis, gout, Lyme disease, and unclassified gonarthritis. All patients with RA fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (35). Written informed consent was obtained from all patients.

Cell preparation, staining, and flow cytometry analysis. For the preparation of leukocytes, freshly obtained synovial fluid was first diluted 1:1 in RPMI 1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (Gibco), and cells were isolated by 2 subsequent centrifugation and washing steps. Synovial fluid cells and whole blood (containing 1 m*M* EDTA) were incubated with the anti-CCR5 monoclonal antibody (mAb) MC-1 (10 μg/ml) or an IgG1 isotype control antibody (10 μg/ml) (MOPC21; Sigma, St. Louis, MO) for 20 minutes on ice. The mAb MC-1 specifically binds to CCR5, as previously shown (36). To determine CCR2 expression, we used the mAb MAB150 (R&D Systems, Minneapolis, MN) and an IgG2b isotype control antibody (MOPC141; Sigma). After 2 washing steps with 0.9% precooled NaCl, cells were incubated for 20 minutes on ice with a fluorescein isothiocyanate–conjugated rabbit anti-mouse $F(ab)_2$ fragment (F313; Dako, Glostrup, Denmark). Cells were washed twice with 0.9% cold NaCl, blocked with 10% mouse serum, and subsequently incubated with a combination of CD4–phycoerythrin (PE) (Leu-3a; Becton Dickinson, Mountain View, CA) and CD8-tricolor (Caltag, South San Francisco, CA), or CD16-PE (Leu-11c; Becton-Dickinson), CD56-PE (Leu-19; Becton-Dickinson), and CD14-tricolor (Caltag). Red blood cells were then lysed and fixed with a semiautomatic device (Coulter, Hialeah, FL) according to the manufacturer's protocol. Cells were immediately analyzed by flow cytometry (Becton-Dickinson) and calculations were performed with Cell Quest analysis software (Becton-Dickinson).

To determine the percentage of CCR5- and CCR2-positive CD4, CD8, or NK cells, lymphocytes were first gated according to their light-scatter properties. A second gate was set on the CD4+, CD8+, or CD16/CD56+ lymphocyte subset, and the number of CCR5- or CCR2-positive cells was calculated after defining a cutoff value according to the isotype control. Monocytes were identified by expression of CD14, and analyzed for CCR5 or CCR2 expression as described above.

Down-modulation of CCR5 from the surface of synovial fluid cells by aminooxypentane (AOP)–RANTES. Freshly isolated synovial fluid cells from additional patients were incubated for 30 minutes with 100 n*M* AOP-RANTES at 37°C or with medium alone as a control. During incubation, cells were resuspended every 10 minutes, and CCR5 expression was determined by flow cytometry analysis as described above and as previously reported (36). In a recent report (36), binding of the MC-1 antibody to CCR5 was not blocked by previous binding of AOP-RANTES to CCR5.

Determination of the CCR5 genotype. Genomic DNA was prepared from frozen blood samples from the patients using a kit from Boehringer Mannheim (Indianapolis, IN). Subsequently, a 274-bp fragment of the CCR5 gene containing the potential 32-bp deletion was amplified by a 40-cycle polymerase chain reaction (PCR) with *Taq* polymerase and a Perkin-Elmer thermocycler (Emeryville, CA). The sequence of the P1 and P2 primers was 5′TTT-ACC-AGA-TCT-CAA-AAA-GAA-G and 5′GGA-GAA-GGA-CAA-TGT-TGT-AGG, respectively. Two PCR fragments, 274 bp and 242 bp in length, were obtained from heterozygous individuals, while in the case of 2 wild-type alleles, only the 274-bp band was detected (Figure 1).

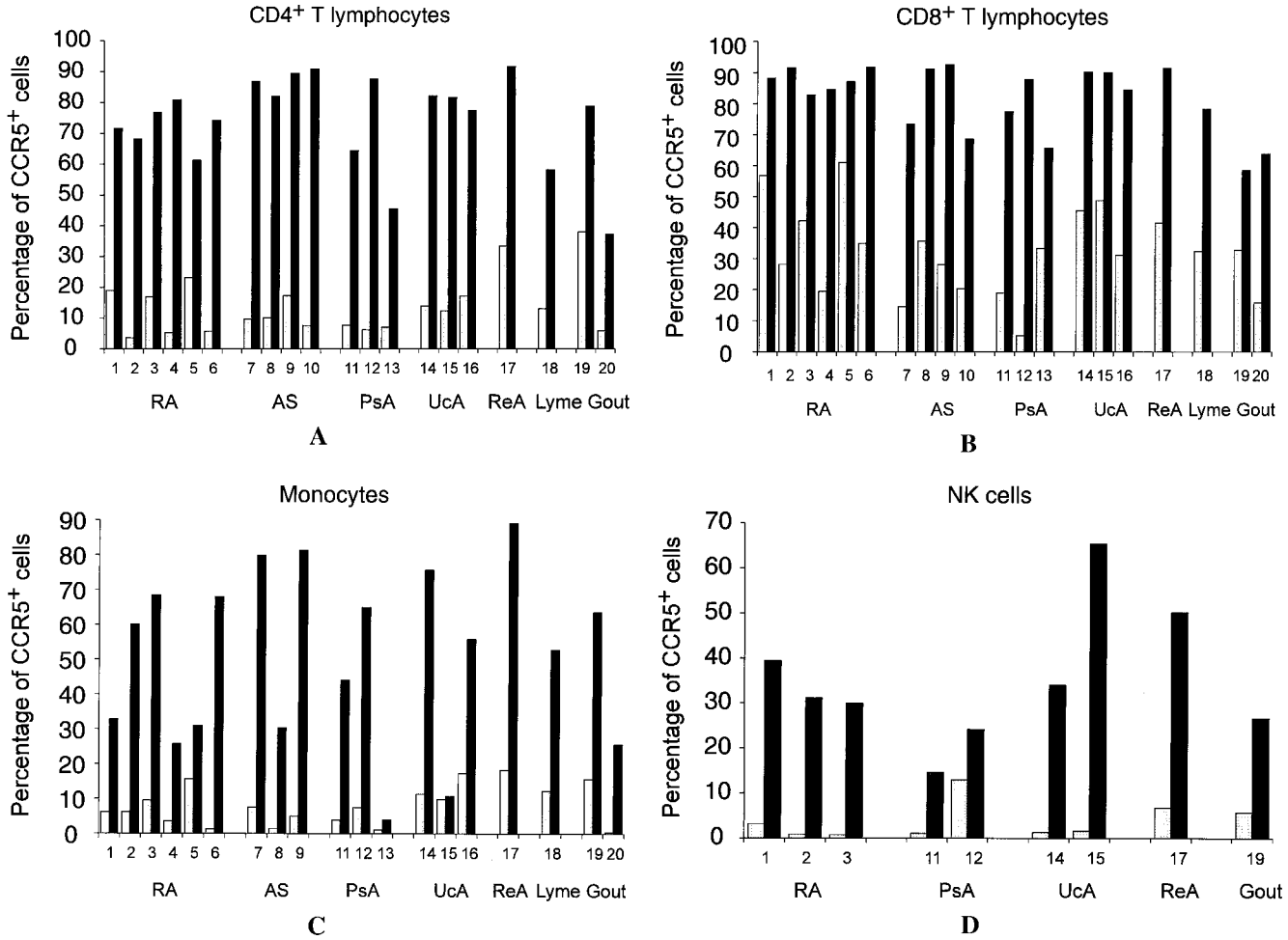

Figure 2. Percentage of CCR5-positive cells in the peripheral blood (shaded bars) and synovial fluid (solid bars) of 20 patients with gonarthritis of different etiology. CCR5-expressing cells were as follows: **A,** CD4+ T lymphocytes; **B,** CD8+ T lymphocytes; **C,** CD14+ monocytes; and **D,** CD56/CD16+ natural killer (NK) cells. RA = rheumatoid arthritis; AS = ankylosing spondylitis with peripheral joint involvement; PsA = psoriatic arthritis; UcA = unclassified arthritis; ReA = reactive arthritis.

RESULTS

For the analysis of CCR5 expression, synovial fluid and blood were obtained from 20 patients with inflammatory joint effusions. The patients' characteristics are shown in Table 1. Six patients presented with RA and 4 with ankylosing spondylitis. Psoriatic arthritis and unclassified arthritis were each diagnosed in 3 patients, gout in 2 patients, and reactive arthritis and Lyme arthritis in 1 patient each. Patients 6, 8, and 19 presented with a disease duration of <1 month, while the others had longstanding arthritis. The percentage of lymphocytes, monocytes, and granulocytes in the synovial fluid, as shown in Table 1, was determined by light microscopy.

We determined the expression of CCR5 on cells isolated from the synovial fluid and compared it with the expression on peripheral blood leukocytes from the same donors. Three-color immunofluorescence was used in flow cytometry analysis to measure the CCR5 expression on CD4+ T cells, CD8+ T cells, monocytes, and NK cells. Monocytes were identified via expression of CD14, while NK cells were identified by staining for CD56/CD16.

In the peripheral blood of all patients, only a small subpopulation of lymphocytes and monocytes expressed CCR5 (Figures 2 and 3). The percentage of CD4+ and CD8+ T cells expressing CCR5 ranged 3.6–38% (mean ± SEM 13 ± 2.1%) and 5.3–61%

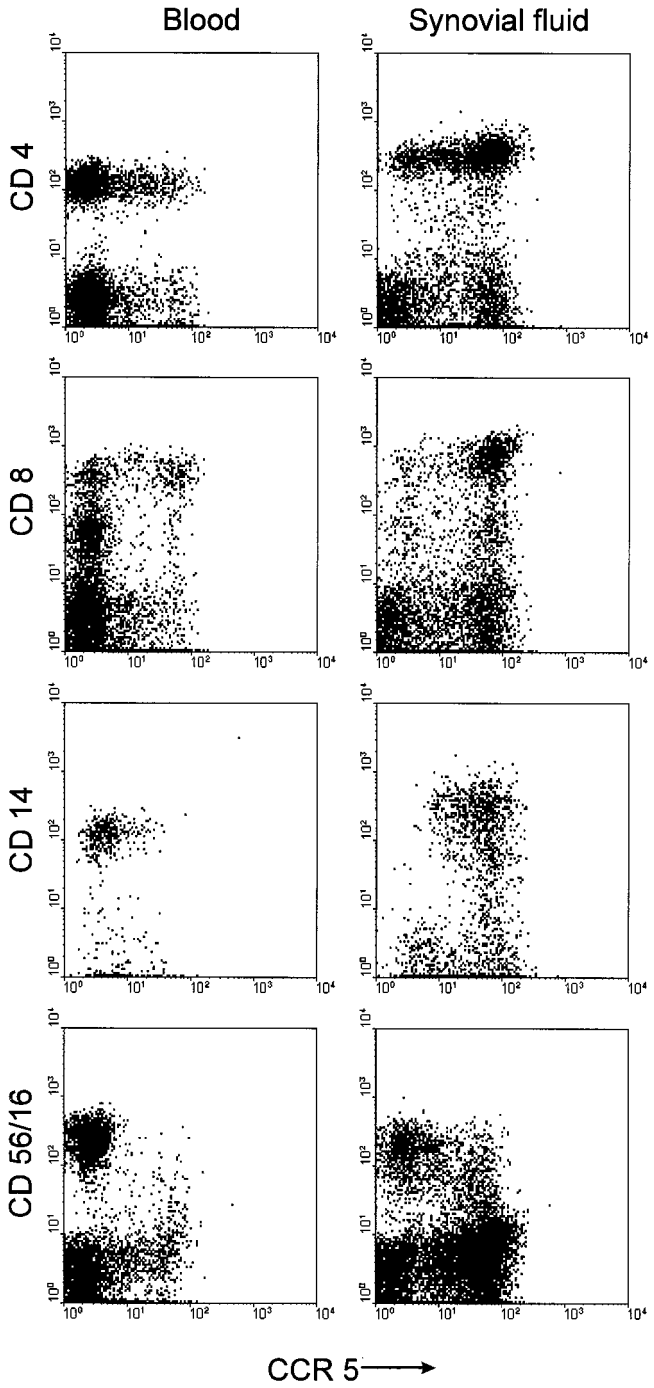

Figure 3. Dot-plot flow cytometry analysis showing CCR5 expression on different cell populations in the peripheral blood and the synovial fluid of a patient with rheumatoid arthritis. Lymphocytes and monocytes were gated according to their light-scatter properties. Three-color immunofluorescence was used to determine CCR5 expression (horizontal axis) and the expression of CD4, CD8, CD14, and CD16/CD56 (vertical axis).

(mean ± SEM 32 ± 3.2%), respectively. Moreover, CD4+ T cells showed a lower CCR5 signal than did CD8+ T cells. In the peripheral blood, only a small subpopulation of monocytes expressed CCR5 (mean ± SEM 7.8 ± 1.4%).

In contrast, an impressive predominance of CCR5-positive T cells was found in the synovial effusions, in which 37–92% (mean ± SEM 74 ± 3.3%) of the CD4+ T cells and 59–92% (mean ± SEM 81 ± 2.4%) of the CD8+ T cells expressed CCR5. Similarly, a high percentage of monocytes in the synovial fluid stained positive for CCR5 (mean ± SEM 51% ± 7%) as compared with the peripheral blood. We also found that CCR5 could be expressed by a subset of NK (CD56/CD16) cells. In the peripheral blood, CCR5-positive NK cells were detectable in only 3 of 9 patients. In contrast, in the synovial effusions of all 9 patients examined, a considerable proportion of NK cells expressed CCR5 (range 15–65%, mean ± SEM 35 ± 5%), demonstrating that, in the context of inflammation, CCR5 can also be expressed by NK cells.

The CCR5 expression was equally high in effusions of patients with acute disease and in those of patients with chronic disease. Of interest, 2 patients with gouty arthritis showed a similar predominance of CCR5-positive cells in the synovial fluid. No apparent differences were observed between different forms of arthritis, though this finding has to be verified in a higher number of patients.

In further experiments, we determined the expression of CCR2 on blood and synovial fluid leukocytes from 3 additional patients. Two of these patients had RA and 1 had reactive arthritis. On average, CCR2 expression was found on 22% of CD4+ and on 29% of CD8+ blood T cells, while in the synovial fluid, 54% of the CD4+ T cells and 48% of the CD8+ T cells expressed CCR2. Individual values are shown in Table 2. Monocytes homogeneously stained positive for CCR2 in

Table 2. CCR2-positive CD4+ and CD8+ T lymphocytes in the peripheral blood and the synovial fluid of 2 patients with rheumatoid arthritis and 1 with reactive arthritis*

	Peripheral blood		Synovial fluid	
Patient	CD4+	CD8+	CD4+	CD8+
A	28	36	45	44
B	13	22	46	30
C	25	30	70	70

* Patients A and B had rheumatoid arthritis. Patient C had reactive arthritis. Values are the percentage of CCR2-positive lymphocytes in each subset.

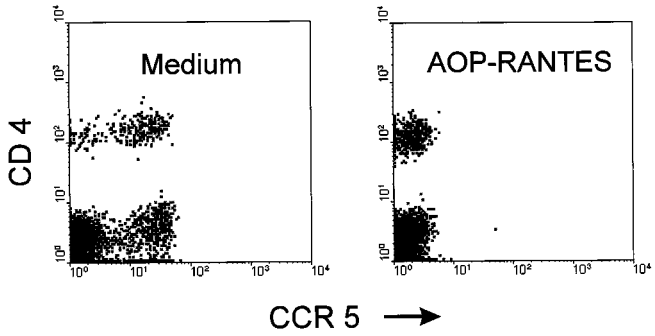

Figure 4. Complete down-modulation of CCR5 on synovial fluid cells by aminooxypentane (AOP)–RANTES. Cells were incubated with medium or AOP-RANTES (100 n*M*) for 30 minutes at 37°C and CCR5 expression was determined on CD4+ and CD8+ T cells by flow cytometry analysis. Staining for CCR5 on the horizontal axis indicates positivity for CCR5.

both compartments, with no difference in the mean fluorescence signal for CCR2.

The CCR5 ligands RANTES, MIP-1α, and MIP-1β have been detected in the synovial fluid of patients with RA. We recently observed that these chemokines induced a concentration-dependent down-modulation of CCR5 from the surface of peripheral blood mononuclear cells. Since we found a higher density of the chemokine receptor CCR5 on cells isolated from the synovial fluid as compared with the peripheral blood (data not shown), we wondered whether CCR5 on synovial fluid cells could be refractory toward down-modulation by chemokines. However, CCR5 could be completely internalized by incubation of cells with AOP-RANTES, an NH_2-terminal modification of RANTES (37) (Figure 4). The latter experiment also served as a further control to exclude nonspecific binding of the CCR5 mAb MC-1 to cells isolated from the synovial fluid.

Because ~20% of the white population is heterozygous for an internal Δ32 deletion in the CCR5 alleles, genotyping was performed and 6 heterozygous patients were identified, i.e., roughly the expected percentage in the white population (Table 1). Interestingly, the predominance of CCR5-positive cells in the synovial fluid did not differ between the CCR5/CCR5 wild-type and the Δ32CCR5/CCR5 heterozygous patients.

DISCUSSION

In this study of 20 patients with various forms of arthritis, we could demonstrate a predominance of CCR5-positive T cells, monocytes, and NK cells in the synovial effusions as compared with the peripheral blood. Although we consistently found a high percentage of CCR5-positive cells in the synovial fluid, there were considerable interindividual differences in the percentage of CCR5-positive cells in the peripheral blood. This variability may reflect systemic activation of cells, due either to the underlying rheumatic disease or to other, as-yet-unknown stimuli. In addition, it is known that a Δ32 mutation in the CCR5 gene leads to a lower CCR5 expression in the peripheral blood (10). By comparing the CCR5 expression in the peripheral blood and the synovial fluid of the same patients, we were able to control for this interindividual variation.

Genotyping of our patients revealed that 6 of 20 individuals were heterozygous for the Δ32CCR5 mutation, a frequency comparable with that reported in the white population (11,12). In 2 recent studies, there was no significantly reduced frequency of Δ32CCR5/CCR5 heterozygous individuals among patients with RA (38,39). Interestingly, we found the same predominance of CCR5-positive cells in the synovial fluid of patients with a heterozygous Δ32CCR5 deletion as in patients with the wild type, indicating a full compensation of the heterozygous phenotype in inflammatory joint effusions.

The notion that the chemokine receptor CCR5 plays a central role in the pathogenesis of RA was challenged by a recent report that described 2 RA patients with a homozygous Δ32CCR5/Δ32CCR5 mutation (40). This finding suggests that a complete deficiency in the CCR5 receptor expression does not prevent the development of RA and that other chemokine receptors (e.g., CCR2, CXCR3) may substitute for the CCR5 deficiency. However, one has to take into account that the immune response of these individuals may have adapted to a deficiency that was present before development of the immune system, and that phenotypic changes might only be observed when the receptor is blocked at later stages.

The marked increase in the percentage of CCR5-positive cells in synovial effusions compared with the peripheral blood might be explained by different mechanisms. It could result either from a preferential attraction of CCR5-positive cells into the inflamed tissues or from the induction of CCR5 expression at the site of inflammation.

The crucial role of chemokines in directing migration of leukocytes has clearly been established in vitro using Boyden chamber or transendothelial migration assays (4,5). In transendothelial migration under flow conditions, only the combined action of adhesion molecules and chemokines enables the transmigration of

cells. In vivo, it is unclear which chemokines and chemokine receptors are primarily responsible for the migration of leukocytes into inflamed tissue. The predominance of CCR5-positive lymphocytes and monocytes in the synovial fluid of patients with arthritis suggests that CCR5 and its ligands could be involved in the pathogenesis of rheumatic joint diseases. However, we cannot conclude that CCR5 is exclusively responsible for immigration of these cells, since other chemokine receptors, together with adhesion molecules, might contribute to inflammatory reactions. A recent report by Qin et al described the prevalence of CXCR3-positive cells in the synovial fluid of 3 patients with RA (24). In contrast to CCR5, however, CXCR3 is expressed on a rather high percentage of peripheral blood T cells, resulting in a low joint-to-blood index for CXCR3-positive cells. The same is true for the chemokine receptor CCR2, for which we found an index of only ~2 for CD4+ and CD8+ T cells. The high joint-to-blood index of ~6 for CCR5-positive T helper cells observed in the present study suggests that in a multistep process of extravasation and migration, the chemokine receptor CCR5 could indeed play a crucial role in the process of inflammation.

In addition, local induction of CCR5 might contribute to the high percentage of CCR5-positive synovial fluid cells. IL-2 has been shown to up-regulate CCR5 expression on T cells after prolonged culture, and IL-10 is able to rapidly induce CCR5 on monocytes (41). Furthermore, monocytes readily express CCR5 after overnight culture, a procedure which might reflect activation and differentiation of monocytes into macrophages. Both IL-2 and IL-10 have been detected in the synovial fluid of patients with arthritis (15).

The expression of CCR5 marks a subset of T cells that is potentially important in the pathogenesis of arthritis. Recently, it was found that CCR5 and CXCR3 are associated with a Th1 phenotype (31,33), while CCR3 and CCR4 are predominantly expressed on the Th2 subset (31,32). Consistent with these findings, a preponderance of Th1 cells was described in the joints of patients with RA and Lyme arthritis (25–29). Little is known about a Th1/Th2 predominance in other types of arthritis.

In further experiments, we investigated whether CCR5 can be down-modulated from the surface of synovial fluid cells in a manner similar to that previously described for peripheral blood T cells and monocytes (36). AOP-RANTES induced a complete internalization of CCR5 from T cells and monocytes in the synovial fluid. The complete down-modulation of CCR5 could be part of a future therapeutic strategy to influence disease progression. Blockade of chemokine receptors by specific antagonists may also help to clarify the role of CCR5 in the pathogenesis of arthritis. Interestingly, in a murine model of arthritis, i.e., collagen-induced arthritis in DBA/1 mice, Met-RANTES, a CC-chemokine receptor antagonist, was able to significantly reduce disease progression (42).

In conclusion, the predominance of CCR5-positive leukocytes in synovial effusions suggests that the chemokine receptor CCR5 could have an important role in the process of joint inflammation. CCR5 might therefore be an interesting target in the treatment of rheumatic joint diseases.

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