Fatal attraction: chemokines and rheumatoid arthritis
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Chapter 7

CHEMOKINE BLOCKADE IN CHRONIC INFLAMMATORY DISEASE: PROOF OF CONCEPT IN PATIENTS WITH RHEUMATOID ARTHRITIS

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ABSTRACT

Objective: Chemokines and their receptors are considered important contributors in cell migration and inflammation in many chronic inflammatory disorders. Chemokines affecting monocytes/macrophages are especially considered potential therapeutic targets, but no studies reporting the effects of blocking the chemokine-repertoire in humans with a chronic inflammatory disease are available yet.

Methods: This study reports a double blind, placebo-controlled, phase -Ib clinical trial with a specific, oral CCR1-antagonist in 16 patients with active rheumatoid arthritis (RA). The primary endpoints of this study were to assess the safety and tolerability of the compound as well as the ACR-20% response and biological markers of synovial inflammation. Patients were randomized 3:1 to active: placebo treatment for 14 days. Clinical evaluation was performed before, during, and after the study. Arthroscopy and synovial biopsies were performed on day 1 and day 15. We used immunohistochemistry to detect CD68+ macrophages, CD4+ T-cells, CD8+ T-cells, CD22+ B-cells, CD138+ plasma cells, CD55+ fibroblast-like synoviocytes, and CCR1+ cells and quantified the results by digital image analysis. The results before and after treatment were compared by the paired t-test. In addition, a two-sample t-test was used to compare the changes from baseline in the two groups.

Results: All patients completed the study. In the synovium there was a significant reduction in the number of macrophages (P=0.016), intimal macrophages (P=0.026), and CCR1+cells (P=0.049) in patients treated with the chemokine antagonist compared to the placebo group. There were also significant decreases in overall cellularity, intimal lining layer cellularity, CD4+T-cells, and CD8+T-cells in treated patients. Cells lacking CCR1 were not affected. Trends towards clinical improvement were observed within the treated patients, but not in the placebo group. Severe side effects were not reported.

Conclusion: These results provide the first evidence that specific chemokine-receptor blockade can result in relevant biological effects in patients with active rheumatoid arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects synovial tissue (ST) in multiple joints. Although its etiology is still unknown, RA is thought of as an autoimmune disease. Severe morbidity and structural damage of joints advocates early and effective treatment (1). Treatment with combinations of disease-modifying anti-rheumatic drugs (DMARDs) can induce clinical remission and may inhibit erosive disease (2). Despite this there is a moderate effectiveness of these agents, and severe side effects as well as poor long-term toleration have been reported (3). This rationalizes the search for more effective and less toxic agents in the treatment of this disease. New targeted therapies, such as tumor necrosis factor (TNF)-α blockade, have improved the therapeutic possibilities, but not all patients respond (4).

Chemokines are a specialized family of small cytokines (8-10 kD) that function as potent mediators of inflammation by their ability to recruit and activate specific leukocyte subpopulations. Several chemokines have been described in RA ST and synovial fluid (SF) including many of the CC-chemokines, among which are CCL3 (Macrophage Inflammatory Protein -1α, MIP-1α) and CCL5 (Regulated-upon-Activation-Normal-T-cell-Expressed-and-Secreted, RANTES) (5-7). Macrophages and T-lymphocytes in the ST are a major source of these chemokines (5:6:8). Chemokines activate leukocytes by binding to specific serpentine G-protein coupled cell-surface receptors on target cells and thus far twelve CC-receptors have
been reported (9:10). CCR1-positive cells are scattered throughout the synovium and the majority of the CCR1-positive cells are macrophages (11). Antibodies to chemokines and chemokine-inhibitors are considered interesting potential therapeutic tools in chronic inflammatory disorders since the discovery of their existence. In vitro experiments showed that blocking of the most powerful ligands of CCR1, CCL3 and CCL5 with neutralizing antibodies could inhibit the chemotactic activity in RA SF (5:6;12:13). In vivo experiments blocking the ligands of CCR1, or antagonizing the CCR1-receptor itself, in animal models of arthritis revealed that pre-treatment prevents inflammation in the synovium and reduces the development of joint destruction as well as the severity of symptoms (14:15). These and other data provide the rationale for the development of a specific CCR1-antagonist as a potential treatment for RA.

In this study chemokine blockade was studied for the first time in humans. The objective was to provide initial evidence of safety and proof of concept. We conducted a double-blind placebo-controlled clinical study with an orally available compound in patients with RA and observed a marked decrease in synovial inflammation as well as trends towards decrease in clinical disease activity after treatment for only two weeks.

**PATIENTS AND METHODS**

**Patients.**

In a period of 8 months 16 patients, aged 18-80, were included if they met the criteria of the American College of Rheumatology (ACR) for the diagnosis of RA (16). We included patients with a disease duration of at least 6 months and active disease despite possible current medication. Patients were allowed to use certain DMARDs (methotrexate [no more than 15 mg/week], hydrochloroquine, or sulfasalazine), provided such treatment had been without significant renal, hepatic, gastro-intestinal, hematologic, or dermatological toxicity, and the dose/schedule had been stable for at least two months prior to entry. Patients were also allowed to receive corticosteroid therapy equivalent to an average dose of prednisone ≤ 10 mg/day, provided that the dosage had been stable for at least two months prior to entry. Non-steroidal anti-inflammatory drugs were allowed, provided the dose and frequency had been stable for 30 days. Patients were excluded if they had severe physical incapacity (Steinbrocker class IV) (17). Other exclusion criteria were: concomitant requirement for an immunosuppressive agent such as azathioprine or cyclosporine: first manifestation of RA before 16th birthday: clinically significant concurrent neurologic, hematologic, renal, hepatic, endocrine, pulmonary, or cardiovascular disease (i.e. not controlled by a stable therapeutic regimen), concomitant therapy with another investigational drug in the past 30 days: any screening laboratory deviations more than 30% from upper or lower limits of the normal range for liver function tests, white blood cell counts without normal limits, hemoglobin and hematocrit outside ranges typical for a RA patient (i.e. ≥30 %). severe thrombocytopenia (≤100 10E9/L), thrombocytosis (≥750 10E9/L) or abnormal renal function tests (i.e. BUN or creatinine more than 10 % above the upper limits of normal). Female patients of childbearing potential used effective methods of birth control and had a negative pregnancy test before entry. Pregnant or nursing mothers were not included.
Table 1. Clinical data.

<table>
<thead>
<tr>
<th>Measure of activity</th>
<th>Placebo (n=4) §</th>
<th>CCR1 Antagonist (n=12) §</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before (mean±sem)</td>
<td>After (mean±sem)</td>
</tr>
<tr>
<td>Tender Joint Count</td>
<td>10 ±3</td>
<td>7 ±2</td>
</tr>
<tr>
<td>Swollen Joint Count</td>
<td>13 ±2</td>
<td>10 ±2</td>
</tr>
<tr>
<td>Pain (VAS)*</td>
<td>8 ±2</td>
<td>12 ±3</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>26 ±11</td>
<td>31 ±12</td>
</tr>
<tr>
<td>Quality of life (HAQ)*</td>
<td>1.313 ± 0.514</td>
<td>1.219 ± 0.434</td>
</tr>
<tr>
<td>SGADA *</td>
<td>3 ±0</td>
<td>3 ±0</td>
</tr>
<tr>
<td>PGADA *</td>
<td>3 ±0</td>
<td>3 ±0</td>
</tr>
<tr>
<td>DAS*</td>
<td>4.69 ± 0.54</td>
<td>4.60 ± 0.47</td>
</tr>
<tr>
<td>ACR20%*</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

§ For patients who received intraarticular corticosteroids on day 15 (n=4) the clinical data of day 15 (before corticosteroids) were used instead of the day 18 data.

* VAS denotes Visual Analogue Scale. 0 is best and 100 is worst. HAQ denotes Health Assessment Questionnaire, on this scale 0 is best and 3 is worst. SGADA denotes Subjects Global Assessment of Disease Activity and PGADA denotes Physicians Global Assessment of Disease Activity, on these scales 0 is best and 5 is worst. DAS denotes Disease Activity Score for 28 joints and ACR 20% denotes the number of subjects responding to the ACR 20% response criteria.

# The paired-T-test was used to determine significant differences within each treatment group. There were no statistical differences in change between the placebo group and the CCR1 antagonist group.

Study Protocol.
The Medical Ethics Committee of the Academic Medical Centre from the University of Amsterdam approved the study protocol. All patients were included in this centre. Before start of the study, patients gave written informed consent, had a complete medical history taken and underwent a full physical examination. Laboratory examination on hematology and serum chemical profile and analysis of a clean-catch urine specimen were also completed as well as an EKG. Patients had active disease, which was defined by the presence of at least 3 of the 4 following characteristics: the presence of at least 6 or more painful/tender joints, 3 or more swollen joints, at least 45 minutes of morning stiffness and CRP ≥ 0.2 mg/dl. After this initial screening, patients were, if eligible, included in the study within 4 weeks. Clinical assessment for disease activity was repeated at baseline (prior to the arthroscopy), day 8, day 15 and at day 18. This included a 28-joint count for joint swelling and tenderness, physician’s and patient’s assessment of disease activity on a scale from 0 (asymptomatic) to 5 (severe symptoms), pain assessed by a visual analogue scale (VAS) from 0 (no pain) to 100 (severe pain), quality of life (HAQ) from 1 (no disability) to 5 (severe disability) and the erythrocyte sedimentation rate (ESR). The same, blinded, independent assessor who was also blinded for laboratory results performed clinical evaluation. Monitoring for adverse events occurred daily.
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during the study and thereafter by interviews, physical examination and laboratory testing. After cessation of treatment on day 15, patients were seen for follow-up on day 18 and followed up for another 4 weeks.

Table 2. Data on synovial tissue analysis before (mean ± sem) and after (mean ± sem) treatment and the percentual change (mean ± sem) in the placebo group and the verum group compared to baseline. Data represent total cell count in 18 high power fields corrected for the percentage of actual tissue in the analyzed areas for cellularity, CD68+ macrophages, CD4+ lymphocytes, CD8+ lymphocytes, CCR1+ cells, CD22+ lymphocytes, CD55+ fibroblasts and CD138+ plasma cells.

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=4)</th>
<th>CCR1 Antagonist (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before mean (sem)</td>
<td>After mean (sem)</td>
</tr>
<tr>
<td>Overall cellularity</td>
<td>2308 (416)</td>
<td>1741 (414)</td>
</tr>
<tr>
<td>Intimal lining cellularity</td>
<td>553 (141)</td>
<td>401 (79)</td>
</tr>
<tr>
<td>Overall CD68+ macrophages</td>
<td>1915 (339)</td>
<td>2200 (331)</td>
</tr>
<tr>
<td>Intimal lining CD68+ macrophages</td>
<td>900 (201)</td>
<td>1151 (284)</td>
</tr>
<tr>
<td>CD4+ lymphocytes</td>
<td>583 (236)</td>
<td>563 (186)</td>
</tr>
<tr>
<td>CD8+ lymphocytes</td>
<td>175 (96)</td>
<td>153 (25)</td>
</tr>
<tr>
<td>CCR1+ cells</td>
<td>967 (255)</td>
<td>1305 (124)</td>
</tr>
<tr>
<td>CD22+ lymphocytes</td>
<td>630 (359)</td>
<td>668 (184)</td>
</tr>
<tr>
<td>CD55+ fibroblasts</td>
<td>1374 (276)</td>
<td>978 (115)</td>
</tr>
<tr>
<td>CD138+ plasma cells</td>
<td>265 (150)</td>
<td>532 (416)</td>
</tr>
</tbody>
</table>

x represents P value according to the paired t-test for comparison of cell count between baseline and end of study in each group.

* represents P value according to the two-samples t-test for comparison of the percentual change from baseline between the groups.

Study Medication.

A blinded pharmacist, with a ratio of 3:1, randomized patients to receive either the CCR1-antagonist or the placebo. This ratio was chosen in order to optimize power for estimation of CCR1-antagonist treatment related side effects. The CCR1-antagonist was used as an Oral Powder for Constitution (OPC) and was reconstituted in poly-sorbate 80 and sterile water. The placebo OPC mimicking the dose of the CCR1-antagonist was a blend of cellulose, magnesium stearate, sodium lauryl sulfate and was reconstituted with sterile water containing denatonium benzoate and poly-sorbate 80. The characteristics and taste of the CCR1-antagonist and placebo were identical. Treatment was given during 14 sequential days with a dose level of 300 mg (given every 8 hours), based on effects seen in murine models and chemotactic activity in RA SF in an in vitro chemotaxis model.
**Arthroscopy.**

Arthroscopy under local anesthesia was performed in all patients at baseline before treatment and at day 15 after treatment. Arthroscopies, tissue sampling and storage were performed as described previously in detail (18).

**Figure 1.** Significant decrease after CCR1 blockade therapy (---) compared with placebo (-----) in the mean number (sem) of A. overall CD68+ cells (P=0.016), B. intimal lining CD68+ cells (P=0.026) and C. CCR1+ cells (P=0.049) for the CCR1 antagonist group and the placebo group.

![Graphs showing significant decrease](image)

**Immunohistochemical Analysis.**

Serial sections were stained with the following monoclonal antibodies (mAb): anti-CD68 (EBM11, Dako, Glostrup, Denmark), anti-CD3 (SK7, Becton-Dickinson, San Jose, CA), anti-CD4 (SK3, Becton-Dickinson), anti-CD8 (DK25, Dako), anti-CD22 (CLB-B-Ly/1, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), anti-CD138 (IM-2757, Beckman Coulter, Marseilles, France), anti-CD55 (Clone-67, Serotec, Oxford, UK) and anti-CCR1 (MAB145, R&D systems Europe Ltd., Abingdon, UK). Sections with non-assessable tissue, defined by the absence of an intimal lining layer, were omitted before analysis. For control sections, the primary antibodies were omitted or irrelevant isotype-matched mouse antibodies were applied (anti-human immunodeficiency virus, a gift from TNO, Rijswijk, The Netherlands). Staining was performed according to a 3-step immunoperoxidase method as previously described (19).

**Digital Image Analysis.**

The slides were evaluated by digital image analysis. All sections were coded and analysed in a random order by an independent observer (JJH) who was blinded for the clinical data as described previously (20).
Statistical Analysis.
The results before and after treatment were compared by the paired t-test. In addition, a two-sample t-test was used to compare the changes from baseline in the two groups. The authors performed data acquisition, data entry, database management and data analysis.

RESULTS
Demographic Features.
Four men and twelve women were included in the trial. Their mean age was 53 years and all patients had active disease despite using DMARDs. In the placebo group there was one male and three females with a mean age of 50 years (range 25-61 years) and in the CCR1 antagonist treated group there were 3 males and 9 females with a mean age of 57 years (range 43-79 years). The patients in the placebo group had a mean disease duration of 42 months: mean disease activity score (DAS) of 4.69 (range 3.43-6.06) and 50 percent of the patients was rheumatoid factor positive. The patients in the CCR1 antagonist group had a mean disease duration of 87 months (range 6-336 months); a mean DAS of 5.58 (range 4.31-6.95) and 58 percent of these patients was rheumatoid factor positive. With regard to DMARD therapy, all patients in the placebo group used methotrexate and one of them also used low dose prednisone. In the CCR1 antagonist group 9 patients used methotrexate (three in combination with hydrochloroquine or low dose prednisone), two patients only used sulphasalazine and one patient only used hydrochloroquine. There were no significant differences in age, sex ratio, disease duration and concomitant use of DMARDs, but patients in the treated group were on average 7 years older and had 45 more months disease duration. All patients had active disease at entry, as judged by the presence of multiple tender and swollen joints, pain assessed on a visual analogue scale, raised acute phase reactants, morning stiffness, subject’s as well as physician’s global assessment of disease activity and as calculated in the disease activity score (DAS) (21). Comparison of the clinical and laboratory indices of disease activity between the 2 groups on day of entry revealed only higher scores for tender and swollen joints in the treated group.

Safety and Tolerability.
The compound was well tolerated and all 16 randomized patients completed the study. There were no severe adverse events reported. Adverse events related or potentially related to the compound were nausea after medication intake (n=1), which also occurred in the placebo group (n=1) and might be explained by the bitterness of the OPC. Mild transient ankle edema (n=1) and a resolving rash (n=1) were also reported in the treated group. No major abnormalities in hematological findings or serum chemical findings were noted during or after the study.

Clinical Efficacy.
The characteristics of the clinical efficacy are summarized in Table 1. Four patients (1 patient from the placebo group and 3 from the CCR1-antagonist group) were non-responders and received intra articular corticosteroids on day 15. The data of day 15 (before receiving corticosteroids) were used for comparison between day 1 and day 18 for these four patients. There were, on average, no significant differences in change between the placebo group and the active treated group. The treated group did show statistically significant clinical improvement in the number of tender joints (P=0.021), swollen joints (P=0.001), quality of life (HAQ, P=0.037) and in the disease activity score (P=0.012) after treatment. Whereas in the placebo group there was no significant clinical improvement in any of the parameters.
According to the ACR response criteria (22) at day 18, 4 patients (33%) treated with the CCR1 antagonist had at least 20% improvement, but none of the controls.

Figure 2. Expression of overall-CD68+ cells (A), CD68+ lining cells (B) and CCR1+ cells (C) in paired synovial biopsies after CCR1 blockade (---) or placebo (---) for the individual patients.

**Immunohistochemical Analysis.**

The results of the immunohistochemical analysis at baseline and after 2 weeks of treatment are depicted in Table 2. Staining was negative in control sections, where the primary antibody was omitted or irrelevant antibodies were applied. After CCR1 blockade there was on average a decrease in the treated group in the number of CD68+ macrophages of 34% (sem 4%) (P=0.016), intimal lining layer CD68+ macrophages of 41% (sem 5%) (P=0.026) and CCR1+ cells of 66% (sem 84%) (P=0.049), compared to the placebo group where there was on average a decrease of 15% (sem 5%), 28% (sem 13%) and an increase of 35% (sem 5%), respectively (Figures 1 and 2). When the changes within each treatment group were compared before and after treatment there was also a significant reduction in the treated group in overall cellularity (reduction from 1795 ±256 (mean ± sem) to 967 ±164 (P=0.013), intimal lining layer cellularity (423 ±95 to 209 ±49) (P=0.048), CD4+ T-cells (666 ±160 to 358 ±139) (P=0.023) and CD8+ T-cells (157 ±44 to 84 ±31) (P=0.024). There was no significant change in any of the measured cell types in the placebo group. The number of CD22+ B-cells, CD138+ plasma cells and CD55+ fibroblast-like-synoviocytes did not change in either group, suggesting that only cells that are capable of expressing CCR1 were affected. Representative examples of immunohistochemical staining before and after treatment are...
shown in Figures 3, 4 and 5. Figure 6 shows an example of an isotype specific negative control.

**DISCUSSION**

The results of this study show that, in a short-term treatment, chemokine receptor blockade appears a safe and potentially effective therapy for patients with a chronic inflammatory disease. This confirms the importance of chemokines and chemokine-receptors in cell trafficking and offers a completely new approach for the treatment of these disorders. This study describes for the first time the pronounced effect of chemokine receptor blockade on the features of ST in RA patients.

To date none of the available therapies is curative for RA and despite the optimal use of current anti-rheumatic therapy there still is a need for better treatments. In the search for more effective targeted therapies, new strategies aimed at blocking key mediators in the inflammatory process have been described (4:23). This has stimulated the search for new biological molecules that could be targeted in order to treat RA in a safe and effective manner. There is especially a need for targeted small molecules, which have the advantage that they can be taken orally.

**Figure 3.** Representative synovial expression of CD68+ macrophages before and after treatment for a placebo patient (A,B) and a treated patient (C,D). Original magnification x400. A full colour image of figure 3 is provided in the Appendix (Chapter 7, figure 3).

Chemokines and their receptors play a central role in the recruitment of leukocytes into the inflamed tissue and the perpetuation of inflammation (24:25). Numerous studies described the presence of chemokines and their receptors in the synovial compartment of patients with RA and other forms of arthritis (11:25-28). CCR1 and its ligands CCL2, CCL3, CCL4 and CCL5 have been implicated in the pathogenesis of RA and other chronic inflammatory diseases (29:30). It has been suggested that CCR1 is involved in the initial recruitment of monocytes to sites of inflammation (11). Successful blocking of the receptor and its ligands has been shown in *in vitro* models and in animal models for arthritis (6;12-15;31).
We conducted a placebo-controlled, double blind, randomised study to provide initial proof of concept. We studied 12 CCR1 antagonist treated patients and 4 placebo patients. A limitation of this study is the small study population, especially the number of control patients. To interpret the results it is important to note that previous work has shown that analysis of serial STT samples from RA patients, who received either placebo or unsuccessful treatment with recombinant human interleukin-10 did not reveal any synovial changes (32).

**Figure 4.** Representative synovial expression of CD4+ lymphocytes before and after treatment for a placebo patient (A,B) and a treated patient (C,D). Original magnification x400. A full colour image of figure 4 is provided in the Appendix (Chapter 7, figure 4).

Similarly, there was no clear-cut change in serial biopsies after treatment with IL-1Ra at 30 mg/day (33), which appears to have very limited effects on arthritis activity. Hence, this supports the view that it is unlikely that changes in serial biopsy samples can be explained by placebo effects, regression to the mean, expectation bias, or by the arthroscopy procedure itself, but they reflect biological effects of the treatment. Consistent with this notion, on average, we did not observe any decrease in synovial inflammation in the 4 patients who received placebo.

**Figure 5.** Representative synovial expression of CCR1+ cells before and after treatment for a placebo patient (A,B) and a treated patient (C,D). Original magnification x400. A full colour image of figure 5 is provided in the Appendix (Chapter 7, figure 5).
In synovial samples from patients treated with the CCR1 antagonist there was a significant decrease in the overall number of macrophages as well as in intimal lining layer macrophages and CCR1+ cells when the treated group was compared to the placebo group. There also was a significant reduction in overall cellularity, intimal lining layer cellularity, CD4+ T-cells, and CD8+ T-cells in the treated group. Cells not capable of expressing CCR1 (including CD22+ B-cells, CD138 plasma cells, and CD55+ fibroblast-like-synoviocytes) were not affected by the treatment. These results confirm the feasibility and specificity of CCR1 blockade in vivo. It should be noted, however, that synovial inflammation could occasionally be reduced in the absence of clinical improvement (19). Another study revealed synovial changes in patients with only a modest decrease in serum levels of acute phase reactants (34). Taken together, these studies indicate that analysis of serial biopsies can be used as a screening method to test new compounds requiring relatively small numbers of patients.

**Figure 6.** Isotype-specific negative control. Original magnification x200. A full colour image of figure 6 is provided in the Appendix (Chapter 7, figure 6).

The clinical effects described in this study appear promising, even after short treatment. It should be noted that there were some differences between the two groups with regard to age, disease duration and disease activity. The patients in the CCR1 antagonist group were on average a bit older, had disease of longer duration, and tended to have more disease activity at baseline. It appears unlikely that these differences influenced the clinical response to treatment. It is obvious, however, that a meaningful clinical effect needs to be shown in larger, well-controlled studies. This study provides the rationale for such trials.

The present study shows proof of principle of CCR1 blockade in patients with chronic inflammatory disease. Administration of an oral CCR1 antagonist resulted in a striking decrease in synovial inflammation. This may provide a completely new direction in the treatment of chronic inflammatory disorders and encourages future investigations and clinical trials aimed at inhibition of other important members of the chemokine repertoire, such as CCR1, CCR5, CCR2, CXCR3, MCP-1 and others (11;24;26).

**Reference List**


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