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# INHIBITION OF NEUTROPHIL MIGRATION SOON AFTER INITIATION OF TREATMENT WITH LEFLUNOMIDE OR METHOTREXATE IN PATIENTS WITH RHEUMATOID ARTHRITIS

## Findings in a Prospective, Randomized, Double-Blind Clinical Trial in Fifteen Patients

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*Objective*. **Leflunomide is a novel immunomodulating drug that has recently been approved as a disease-modifying antirheumatic drug for the treatment of rheumatoid arthritis (RA). The aim of this study was to determine the relationship between the clinical effects of leflunomide and neutrophil migration.**

*Methods*. **The effects of leflunomide and methotrexate on neutrophil chemotaxis were studied in 15 RA patients who participated in a prospective, randomized, double-blind clinical trial. When possible, neutrophil numbers were counted in synovial fluid (SF) samples at baseline and after 14 days, 4 months, and 1 year of treatment. The chemotactic properties of peripheral blood neutrophils from RA patients treated with either leflunomide or methotrexate were studied by the Boyden chamber technique, using the activators formyl-methionyl-leucyl-phenylalanine (fMLP) and interleukin-8 (IL-8). The in vitro effects of A77 1726, the active metabolite of leflunomide, and methotrexate on peripheral blood neutrophils from 7 healthy control subjects were also investigated.**

*Results*. **Both therapy groups exhibited clinical improvement, including rapid reductions in SF neutrophil counts and reduced joint swelling and tenderness. On day 14, 3 of 7 patients who received leflunomide showed no detectable effusions. There was a significant effect on neutrophil chemotaxis ($P < 0.001$), which was similar for leflunomide and methotrexate. The direct effects on the neutrophils diminished over time. Incubation of peripheral blood neutrophils from healthy controls with A77 1726 confirmed the inhibitory effect on chemotaxis.**

*Conclusion*. **Leflunomide treatment is beneficial in RA patients. Different mechanisms are operative in various phases of treatment, leading to decreased recruitment of inflammatory cells in the joints.**

Leflunomide is a novel immunomodulating agent that has recently been approved by the United States Food and Drug Administration as a disease-modifying antirheumatic drug (DMARD) for the treatment of patients with rheumatoid arthritis (RA). In phase III clinical trials, leflunomide showed efficacy comparable to that of sulfasalazine and methotrexate, with a favorable safety profile (1,2). After oral administration, leflunomide is rapidly converted by the opening of the isoxazole ring into the active metabolite A77 1726.

The exact mechanism by which A77 1726 exerts its effects in vivo is, as yet, unknown. Data suggest two possible modes of action: inhibition of dihydroorotate dehydrogenase (DHODH), by which A77 1726 influences de novo pyrimidine biosynthesis (3–6), and interaction with primary and secondary signaling events by interference with the phosphorylation of tyrosine kinases (7,8). The inhibition of de novo pyrimidine biosynthesis by A77 1726 is ~100-fold stronger than its effects on tyrosine kinase (3). Since these effects on cell

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signaling and proliferation can be reversed by washing procedures, it appears that A77 1726 acts predominantly as a cytostatic, and not as a cytotoxic, drug (9). In vitro experiments with A77 1726 have shown effects on B cell function (10), T cell proliferation (11), and neutrophil function (12). The documented effect of A77 1726 on neutrophil function involves regulation of mediators, such as arachidonic acid metabolites (13). Together with lysosomal enzymes and toxic oxygen derivatives, these mediators have deleterious effects that lead to tissue injury at sites of neutrophil accumulation (14).

In RA, the neutrophil plays an important role in the pathogenesis of synovial inflammation (15). This cell type may constitute more than 90% of the cellular exudate in the synovial fluid (SF) (16) and is present in the inflamed synovial tissue at the interface of cartilage with pannus (17). Methylprednisolone (18) and clinically effective DMARDs, such as gold (19–21) and sulfasalazine (20), can modify neutrophil function. Data on the effects of methotrexate on neutrophil function vary with regard to its inhibitory effects on neutrophil chemotaxis (20,22,23).

To determine whether the clinical effects of leflunomide could be attributed in part to an inhibitory effect on neutrophil migration into the inflamed joint, we studied the effects of this compound on neutrophil numbers in SF from RA patients who were treated with leflunomide compared with methotrexate. Furthermore, experiments were performed to provide insight into the chemotactic properties of peripheral blood neutrophils from the treated patients. Finally, we analyzed the effects of A77 1726 on peripheral blood neutrophils from normal control subjects in vitro.

## PATIENTS AND METHODS

**Patients and controls.** Fifteen patients with active RA according to the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 criteria (24) were included in a prospective, randomized, double-blind clinical trial comparing leflunomide with methotrexate. Active disease was defined as ≥6 swollen and ≥6 tender joints, and patient's and physician's assessments of disease activity as moderate or worse. After a washout period of 28 days, 7 patients received 20 mg of leflunomide/day (after a loading dose of 100 mg/day for 3 days), and 8 patients received methotrexate 7.5 mg/week, which was increased stepwise to 15 mg/week. All patients used concomitant stable doses of nonsteroidal antiinflammatory drugs (NSAIDs) and 1 patient in the methotrexate group took low-dose prednisone (5 mg/day).

Clinical assessment was performed by 1 observer (MCK) at baseline and after 14 days, 4 months, and 1 year. Assessments included a tender joint count (TJC; 28 joints counted, including both knee joints), swollen joint count (SJC; 28 joints counted, including both knee joints), and the patient's and investigator's global assessments of disease activity. The C-reactive protein (CRP) level was assessed as a measure of inflammation. Clinical and laboratory parameters were used to assess improvement according to the ACR criteria (25).

Joint aspiration was performed on the same knee joint at baseline and after 4 months. When possible, SF sampling was performed after 14 days and 1 year. The number of neutrophils was counted in these SF samples. For the chemotaxis assay, blood samples were drawn by venipuncture at baseline and after 3 days, 14 days, 4 months, and (in 6 patients) 1 year of treatment.

Neutrophils from 7 healthy volunteers were used as negative controls. These cells were incubated with various concentrations of A77 1726 (0.1–200 $\mu M$). All study subjects gave informed consent, and the Medical Ethics Committee of the Leiden University Medical Center approved the study protocol.

**Reagents.** Formyl-methionyl-leucyl-phenylalanine (fMLP) was purchased from Sigma (St. Louis, MO), interleukin-8 (IL-8) from R & D Systems Europe (Abingdon, UK), and all other chemicals, which were of the highest purity available, were obtained from Sigma. A77 1726, the active metabolite of leflunomide, was obtained in powder form (kindly provided by Dr. R. R. Bartlett, Hoechst Marion Roussel, Wiesbaden, Germany), and dissolved in 0.01*M* DMSO (Amresco, Solon, OH). The A77 1726 solutions were freshly prepared on the day of the experiment and diluted to appropriate concentrations. Equal concentrations of DMSO were used in all controls and experiments with methotrexate.

**Cell isolation.** We used 2 methods for determining neutrophil chemotaxis. In the first method, 10 cc of blood was collected into tubes containing heparin (Becton Dickinson, Bilthoven, The Netherlands). The sample was subsequently diluted with a 4-fold volume of heparinized medium, and layered on top of Ficoll-amidotrizoate (density 1.077). After centrifugation (20 minutes at 580*g*), the pellet was resuspended in 5 ml of heparinized medium, and starch (6% poly[*O*-2-hydroxyethyl]starch in 0.9% NaCl; 4 ml) was added to sediment the erythrocytes. The resulting neutrophil-containing supernatant was subsequently centrifuged (3 minutes at 480*g*), the remaining erythrocytes were removed by hypotonic hemolysis, and the cells were suspended in medium (140 m*M* NaCl, 5 m*M* KCl, 10 m*M* glucose, 0.5% bovine serum albumin [BSA], and 20 m*M* HEPES, pH 7.3). The cells consisted of >95% neutrophils, of which >99% were viable, as determined by trypan blue exclusion. Unless otherwise stated, the medium was supplemented with 1 m*M* $Ca^{2+}$ and 1 m*M* $Mg^{2+}$ during the experiments. The final cell suspension during the experiments contained $3 \times 10^6$ neutrophils/ml.

In the second method, whole blood was used, as described previously (26,27). Briefly, 10 cc of heparinized blood was diluted with 10 cc of medium (140 m*M* NaCl, 5 m*M* KCl, 10 m*M* glucose, 0.5% BSA, and 20 m*M* HEPES, pH 7.3). Unless stated otherwise, the medium was supplemented with 1 m*M* $Ca^{2+}$ and 1 m*M* $Mg^{2+}$ during the experiments.

**Chemotaxis assay.** Cell migration was measured using the Boyden chamber technique as modified by Zigmond (28). This technique allowed us to measure neutrophil migration directed toward an activator and to compare that with random migration. A cellulose acetate Millipore filter (pore size 3 $\mu$m;

**Table 1.** Demographic data for the study patients*

| | Leflunomide (n = 7) | Methotrexate (n = 8) |
|---|---|---|
| Age, years | 63 (45–79) | 66 (51–78) |
| Sex, M/F | 3/4 | 5/3 |
| Disease duration, months | 33 (6–59) | 133 (5–314) |
| Previous DMARDs | 1 (0–1) | 2 (0–4) |

* Except for the number of males/number of females, values are the mean (range). DMARDs = disease-modifying antirheumatic drugs.

Millipore, Bedford, MA) separated the 2 compartments of the chamber. One of the activators fMLP or IL-8 (29) or medium was placed in the lower compartment, while either isolated neutrophils or 50% diluted whole blood was placed in the upper compartment of the chamber, followed by incubation for 35 minutes at 37°C.

After migration, the filters were fixed and stained, and the distance traveled into the filter (in micrometers) was determined according to the leading-front technique. Chemotactic assays were carried out in duplicate, and the migration distance of the neutrophils was determined at 5 different filter sites. Differences between duplicates never exceeded 10%. In all experiments, the investigator (BMK) was blinded as to the treatment, clinical details, and visit number.

**Electroporation procedures.** Isolated neutrophils from healthy control subjects were electroporated according to the method of Grinstein and Furaya (30), with minor modifications. Briefly, neutrophils were exposed to an electric current that temporarily induces pores in the outer cell membrane. The electropermeabilization procedure was carried out at room temperature. Neutrophils ($3 \times 10^6$/ml) in permeabilization medium (135 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 20 m*M* HEPES, pH 7.0, 10 m*M* glucose, and 0.5% BSA) were placed in the cuvette of a Bio-Rad (Richmond, CA) Gene Pulser. The cells were exposed to 2 discharges of 14.75 kV/cm from a 25-mF capacitor. The cell suspension was stirred between the 2 discharges.

**Statistical analysis.** All mean values for the chemotaxis assays for each RA patient are the arithmetical means of 5 different filter sites of a duplicate experiment; those for the healthy controls are the arithmetical means of 3 different experiments. To compare the effects of leflunomide with those of methotrexate on the migration of neutrophils from the RA patients, a repeated-measures model was used (31). No assumptions about the variance–covariance matrix were made, and this model has been analyzed with the procedure mixed in the statistical analysis program SAS (32). In this model, time and medication and the interaction between time and medication were used as prognostic variables. The significance of the data for all chemotaxis experiments was calculated with the nonparametric Wilcoxon signed rank test for paired data. All clinical data are shown without further statistical analysis.

## RESULTS

**Patient demographics and baseline characteristics.** Demographic and clinical data on the 15 RA patients included in the clinical study are shown in Table 1. The sex and age distribution was similar in the leflunomide- and methotrexate-treated patients. Both treatment groups contained patients with recent-onset disease and longstanding disease, although the methotrexate group tended to have longer disease duration and to have used more DMARDs before the start of the clinical trial.

**Clinical response and SF analysis.** As shown in Table 2, there was active arthritis in multiple joints at baseline. At 4 months, the leflunomide–treated patients showed 67%, 47%, and 54% changes in the TJC, SJC, and CRP values. Corresponding numbers for the methotrexate–treated patients were 29%, 25%, and 18%, respectively. After 1 year of treatment, leflunomide and methotrexate showed similar effects.

After 14 days of treatment, SF was undetectable by clinical examination (confirmed by a "dry tap" on aspiration) in 3 of the 7 patients who received leflunomide, but SF was present in all of the 8 patients who

**Table 2.** Clinical data for the study patients*

| | Leflunomide | | | Methotrexate | | |
|---|---|---|---|---|---|---|
| | Baseline (n = 7) | 4 months (n = 7) | 1 year (n = 6) | Baseline (n = 8) | 4 months (n = 8) | 1 year (n = 7) |
| Tender joint count | 18 ± 3.2 | 6 ± 1.3 | 4 ± 1.6 | 17 ± 3.2 | 12 ± 3.6 | 4 ± 1.4 |
| Swollen joint count | 15 ± 2.5 | 8 ± 1.4 | 6 ± 1.6 | 16 ± 1.7 | 12 ± 2.4 | 7 ± 1.9 |
| CRP (mg/liter) | 74 ± 32.7 | 34 ± 24.0 | 17 ± 4.9 | 49 ± 17.1 | 40 ± 23.8 | 20 ± 11.0 |
| Physician's assessment of disease activity | 3.2 ± 0.18 | 2.3 ± 0.18 | 2.7 ± 0.21 | 3.5 ± 0.19 | 2.4 ± 0.26 | 2.4 ± 0.20 |
| Patient's assessment of disease activity | 3.4 ± 0.20 | 2.4 ± 0.30 | 2.8 ± 0.31 | 3.8 ± 0.31 | 2.6 ± 0.26 | 2.6 ± 0.30 |
| Responder status | | | | | | |
| Nonresponder | – | 14 (1) | 0 | – | 13 (1) | 14 (1) |
| ACR 20% response | – | 43 (3) | 17 (1) | – | 62 (5) | 14 (1) |
| ACR 50% response | – | 43 (3) | 83 (5) | – | 25 (2) | 72 (5) |

* Values are the mean ± SEM, except for the responder status values, which are the percentage (no. of patients). CRP = C-reactive protein; ACR = American College of Rheumatology.

**Table 3.** Neutrophil counts (*10E9/l) in the synovial fluid of 7 RA patients treated with leflunomide and 8 RA patients treated with methotrexate*

| Treatment, patient | Baseline | 14 days | 4 months | 1 year |
|---|---|---|---|---|
| Leflunomide | | | | |
| 1 | 6.3 | NA | 8.0 | Dropout |
| 2 | 6.5 | No effusion | 1.4 | No effusion |
| 3 | No effusion | No effusion | No effusion | No effusion |
| 4 | 3.4 | 9.5 | No effusion | Dropout |
| 5 | 0.1 | 1.6 | No effusion | No effusion |
| 6 | 1.3 | NA | No effusion | 0.2 |
| 7 | 11.8 | No effusion | No effusion | No effusion |
| Methotrexate | | | | |
| 1 | 1.9 | NA | No effusion | No effusion |
| 2 | 0.1 | NA | No effusion | No effusion |
| 3 | 3.2 | 2.1 | 1.7 | 3.3 |
| 4 | 2.5 | 1.4 | No effusion | No effusion |
| 5 | 7.5 | 0.9 | 10.7 | Dropout |
| 6 | 0.1 | NA | No effusion | No effusion |
| 7 | 3.4 | NA | No effusion | No effusion |
| 8 | 3.7 | NA | No effusion | No effusion |

* Leflunomide-treated patients 1 and 4, and methotrexate-treated patient 5 discontinued the trial between week 16 and 1 year because of lack of efficacy. RA = rheumatoid arthritis; NA = not available.

received methotrexate (Table 3). However, 2 leflunomide-treated patients and 5 methotrexate-treated patients refused knee aspiration at that time. After 4 months of treatment, 5 leflunomide-treated patients and 6 methotrexate-treated patients showed no signs of knee effusion (dry tap). After 1 year of treatment, SF was again undetectable in most patients from both therapy groups (Table 3). The SF neutrophil counts remained relatively high in patients who did not respond to leflunomide or methotrexate treatment.

**In vitro neutrophil chemotaxis.** In the clinical study, we could not detect any effect on neutrophil migration after isolation procedures. Therefore, we only used the whole-blood method for the evaluation in the clinical study.

The statistical analysis of neutrophil migration

**Table 4.** Data on random neutrophil migration and neutrophil migration toward fMLP and IL-8 in 7 RA patients treated with leflunomide and 8 RA patients treated with methotrexate*

| Treatment, patient | Random migration ($\mu$m) | | | | | fMLP ($\mu$m) | | | | | IL-8 ($\mu$m) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 days | 3 days | 14 days | 4 months | 1 year | 0 days | 3 days | 14 days | 4 months | 1 year | 0 days | 3 days | 14 days | 4 months | 1 year |
| Leflunomide | | | | | | | | | | | | | | | |
| 1 | 47.7 | 38.5 | 48.0 | 44.2 | | 122.3 | 41.6 | 54.3 | 66.9 | | 118.1 | 42.8 | 63.0 | 98.5 | |
| 2 | 45.5 | 44.0 | 40.8 | 43.2 | | 90.7 | 75.5 | 72.1 | 62.9 | | 96.0 | 86.1 | 80.1 | 77.1 | |
| 3 | 40.5 | 50.4 | 41.3 | 40.5 | | 82.4 | 87.6 | 77.9 | 78.9 | | 102.3 | 79.9 | 96.2 | 95.7 | |
| 4 | 39.0 | 39.5 | 38.6 | 39.8 | 39.7 | 83.7 | 78.9 | 79.7 | 84.9 | 87.7 | 94.7 | 86.9 | 87.7 | 94.9 | 95.1 |
| 5 | 40.1 | 40.1 | 40.2 | 41.0 | | 92.7 | 42.6 | 43.9 | 89.2 | | 130.3 | 44.5 | 45.8 | 94.6 | |
| 6 | 38.8 | 37.9 | 37.9 | 38.0 | 37.7 | 95.4 | 94.0 | 92.4 | 93.5 | 94.8 | 94.2 | 92.9 | 88.5 | 90.6 | 97.3 |
| 7 | 45.1 | 42.3 | 43.6 | 45.1 | | 94.2 | 60.5 | 46.5 | 77.7 | | 96.4 | 81.0 | 63.3 | 94.9 | |
| Methotrexate | | | | | | | | | | | | | | | |
| 1 | 51.9 | 41.6 | 45.6 | 45.4 | 45.2 | 82.8 | 47.7 | 52.0 | 63.9 | 73.4 | 90.2 | 41.7 | 45.5 | 84.7 | 95.1 |
| 2 | 48.4 | 48.4 | 45.0 | 42.9 | 42.5 | 92.3 | 61.7 | 49.3 | 74.9 | 77.9 | 99.1 | 98.3 | 71.7 | 92.8 | 95.2 |
| 3 | 40.8 | 43.5 | 44.2 | 44.3 | | 97.4 | 52.4 | 47.5 | 76.2 | | 122.2 | 61.4 | 62.2 | 90.6 | |
| 4 | 42.3 | 41.4 | 42.5 | 40.6 | 40.4 | 97.5 | 42.4 | 46.1 | 78.5 | 81.9 | 99.4 | 53.7 | 58.5 | 84.5 | 88.3 |
| 5 | 46.5 | 43.2 | 44.8 | 44.6 | | 87.0 | 82.1 | 82.6 | 84.1 | | 100.0 | 97.1 | 97.5 | 100.8 | |
| 6 | 37.7 | 36.5 | 37.9 | 37.9 | 37.7 | 76.8 | 46.2 | 50.1 | 79.4 | 81.2 | 88.1 | 74.4 | 53.5 | 97.8 | 99.6 |
| 7 | 42.1 | 41.7 | 40.8 | 44.3 | | 84.9 | 52.8 | 54.2 | 82.7 | | 79.9 | 52.1 | 47.8 | 86.5 | |
| 8 | 41.7 | 41.9 | 40.9 | 40.8 | | 95.5 | 85.1 | 68.4 | 78.1 | | 93.0 | 92.1 | 79.2 | 94.8 | |

* fMLP = formyl-methionyl-leucyl-phenylalanine; IL-8 = interleukin-8; RA = rheumatoid arthritis.

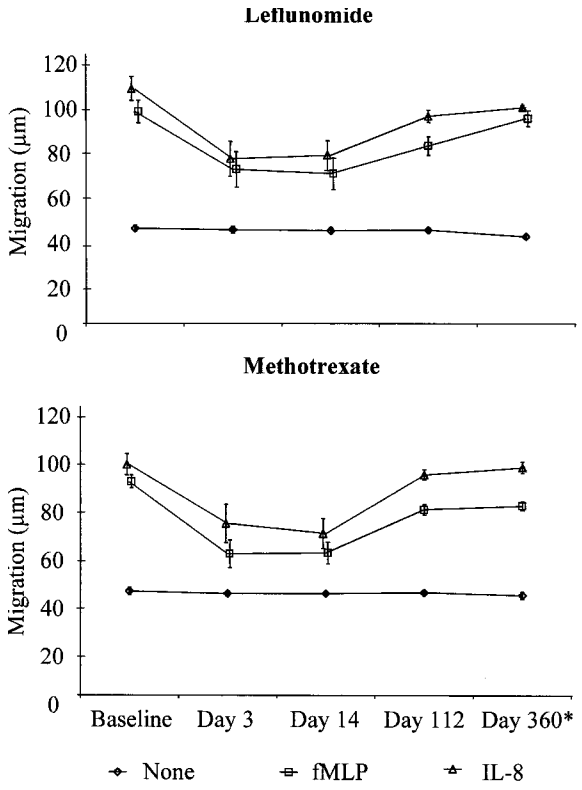


**Figure 1.** Neutrophil migration, as determined in a whole-blood assay without activator, toward formyl-methionyl-leucyl-phenylalanine (fMLP; $1 \times 10^{-9}M$) and interleukin-8 (IL-8; $4 \times 10^{-9}M$) in leflunomide-treated and methotrexate-treated patients at baseline (day 0), and after 3 days, 14 days, 4 months, and 1 year of treatment. * = data on 6 patients (3 leflunomide, 3 methotrexate). Values are the mean ± SEM.

was performed with a repeated-measures model, with time, medication, and their interaction as prognostic variables. No significant interaction was found. Furthermore, this analysis showed that the observed reductions in neutrophil migration over time were highly significant ($P < 0.001$), while no significant difference was observed between leflunomide and methotrexate as the DMARD (confidence interval of difference −4.25, 8.25).

Random neutrophil migration and neutrophil chemotaxis to fMLP and IL-8 were similar in neutrophils from leflunomide- and methotrexate-treated RA patients (Table 4 and Figure 1). By day 3, some of the leflunomide-treated patients already exhibited diminished neutrophil migration toward IL-8 (from a mean ± SEM of 104.6 ± 5.3 μm to 73.4 ± 7.9; $P < 0.02$) and to fMLP (from 94.5 ± 5.0 to 68.7 ± 7.9; $P < 0.01$) (Table 4). Similarly, there was decreased migration toward IL-8 (from 95.9 ± 4.4 to 71.4 ± 7.9; $P < 0.02$) and toward fMLP (from 88.8 ± 2.7 to 58.8 ± 5.8; $P < 0.02$) in the patients who had received methotrexate for 3 days.

On day 14, both treatment groups showed significantly decreased neutrophil chemotaxis to IL-8 (leflunomide group 74.9 ± 6.8 [$P < 0.02$]; methotrexate group 67.2 ± 6.2 [$P < 0.02$]) and fMLP (leflunomide group 66.7 ± 7.0 [$P < 0.02$]; methotrexate group 59.1 ± 4.5 [$P < 0.02$]) compared with baseline. With prolonged treatment, neutrophil migration tended to increase again in both treatment groups (Table 4 and Figure 1). Incubation of neutrophils from both the leflunomide-treated and the methotrexate-treated patients with uridine resulted in restoration of the inhibitory effects on neutrophil chemotaxis.

Incubation of neutrophils from healthy controls with A77 1726 led to a dose-dependent inhibition of neutrophil chemotaxis, up to 50% inhibition of the migration toward fMLP (Figure 2), and up to 70% inhibition of the migration toward IL-8. In the presence of maximal concentrations of A77 1726, neutrophil migration toward fMLP was similar to random migration (mean ± SEM random migration 48.8 ± 1.5 μm; migration in the presence of 200 $\mu M$ A77 1726 47.9 ± 1.3 μm). In contrast, incubation with both A77 1726 and uridine did not affect neutrophil chemotaxis to either fMLP or IL-8. Incubation of neutrophils with uridine alone did not influence neutrophil chemotaxis.

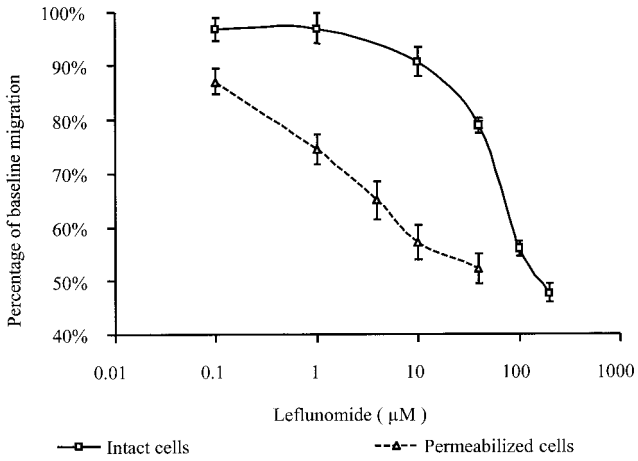


**Figure 2.** Effect of different concentrations of leflunomide (0.1–200 $\mu M$) on neutrophil migration using formyl-methionyl-leucyl-phenylalanine ($1 \times 10^{-9}M$) as the chemotactic agent on intact and permeabilized neutrophils. Values are the mean ± SEM percentage of maximal migration in the absence of leflunomide. Intact cells: The random migration and maximal migration were 48.8 ± 1.5 μm and 100.7 ± 1.5 μm, respectively, for intact cells and 34.6 ± 1.2 μm and 74.2 ± 1.4 μm for permeabilized cells.

After permeabilization of the outer membrane by electroporation, considerably lower A77 1726 concentrations (maximum concentration 40 $\mu M$) were required for the same inhibitory effects compared with intact neutrophils (Figure 2). Prolongation of the preincubation time of neutrophils with A77 1726 up to 3-fold did not alter the effects of A77 1726 on migratory capacity. We could not document the effects of methotrexate on isolated neutrophils from healthy controls in similar experiments as those performed for A77 1726.

## DISCUSSION

This prospective, randomized, double-blind study confirms that both leflunomide and methotrexate are efficacious in the treatment of RA. During the course of treatment, there was a rapid reduction in joint swelling, probably brought about by an early and significant inhibition of neutrophil chemotaxis in RA patients treated with either leflunomide or methotrexate.

Previous studies on the effects of methotrexate on neutrophil function in RA patients have reported both inhibitory (33,34) and miscellaneous (20) effects. We document an absence of effects on neutrophil chemotaxis in leflunomide- and methotrexate-treated patients after isolation procedures, but this could be explained by the washing procedures involved in neutrophil isolation (9,27). The use of a whole-blood assay, excluding washing steps, supports this hypothesis.

Mechanisms by which leflunomide and methotrexate might modify the chemotactic response of neutrophils may involve (a) a direct effect on neutrophil chemotactic receptors, (b) interference with transduction mechanisms of these receptors, or (c) interference with cytoskeletal elements and metabolic processes (35,36). However, the precise mechanisms by which leflunomide and methotrexate exert their action in the neutrophil are, at present, not known.

The neutrophil has negligible amounts of dihydrofolate reductase, the principal target enzyme of methotrexate (37,38), suggesting that mechanisms other than interference with folate metabolism are involved. Reportedly, the inhibition of a polyglutamate derivative of methotrexate is involved in the beneficial effect of methotrexate in RA (35,39,40). This is consistent with the lack of effect of methotrexate in the absence of polyglutamate derivatives of methotrexate, as shown by in vitro experiments (35).

Although the exact intracellular localization of leflunomide is, at present, unknown, the hydrophobic characteristics suggest that the compound is probably present in the phospholipid bilayer of the cell membrane and in cell organelles, such as the mitochondrial membrane. The effects on signaling and pyrimidine metabolism imply that leflunomide may induce its effects not only on the outer cell membrane, but also intracellularly. This notion is supported by the effects of permeabilization of the outer membrane, resulting in direct access of A77 1726 to the intracellular compartment of the cell. After electroporation, 5-fold lower concentrations of A77 1726 produced the same effects on neutrophil chemotaxis. It should be noted that DHODH, through which A77 1726 inhibits pyrimidine synthesis, is predominantly found in the mitochondrial membrane; however, mitochondria are only rarely present in the neutrophil. This suggests that mechanisms other than DHODH inhibition, especially interference with signal transduction processes (8,41), may play a role in mediating the effects of leflunomide on neutrophil chemotaxis.

The effect of isolation procedures on neutrophil chemotaxis, which could completely resolve the inhibiting effects of leflunomide on chemotaxis, is consistent with previous observations that the effects of leflunomide on mononuclear cells are reversible in vitro (9). This could imply that leflunomide has reversible effects on neutrophil homeostasis, as documented by the inhibition of DHODH by leflunomide (42), but which contrasts with the irreversible effects of brequinar sodium (43). In accordance with a study of methotrexate on the inhibition of neutrophil chemotaxis in psoriasis patients (34), the results presented here indicate that the effects of leflunomide on neutrophil chemotaxis are reversible in vivo as well. This could explain the only slightly increased risk of infection in RA patients treated with either leflunomide or methotrexate, since neutrophils are still able to migrate to acutely inflamed regions after prolonged treatment.

The up-regulation of uridine salvage pathways (7) may play an important role in the mitigating effects of leflunomide on neutrophil chemotaxis after extended treatment. This hypothesis is supported by our in vitro data, showing partial restoration of the effects of A77 1726 on RA neutrophils on addition of uridine, whereas the effects were completely compensated in neutrophils from healthy controls.

As stated previously, alternative explanations for the documented effects of leflunomide involve effects on tyrosine kinase activation (7,44) and interference with glycosylation of lipids and proteins that require pyrimidines (45). More speculative is a possible role for uridine as a signaling substance (46). To test this hypothesis,

research should focus on the role of uridine receptors, such as P2 purinergic receptors (46,47), in neutrophil chemotaxis as has been documented for adenine nucleotides (48).

The reduction in joint swelling and tenderness after short-term leflunomide treatment can therefore be explained by a direct effect on neutrophil chemotaxis, resulting in decreased migration of neutrophils into the rheumatoid joints. Since the direct effects on neutrophil chemotaxis diminished after continued treatment, possibly due to the up-regulation of uridine salvage pathways, alternative explanations should be sought for the long-term effects of leflunomide. It is likely that inhibition of cytokine production (49,50) and expression of adhesion molecules (18,51,52) become increasingly important after prolonged treatment. Subsequently, this may lead to a decrease in cell trafficking into the joint. Together with these phenomena, it can be expected that the activation status of neutrophils is reduced in parallel with increased disease control, resulting in changes in the expression of factors, such as tumor necrosis factor $\alpha$ and its receptors (53).

In conclusion, this study shows that the rapid clinical effect of leflunomide treatment in RA patients may be mediated by rapidly decreased neutrophil migration to the synovium. After continued treatment, the effects on cytokine production and expression of adhesion molecules are more likely to be responsible for decreased recruitment of inflammatory cells in the joints.

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