Differential effects of leflunomide and methotrexate on cytokine production in rheumatoid arthritis.
Maarten C Kraan, Tom J.M. Smeets, Marieke J van Loon, Ferdinand C Breedveld, Ben A.C. Dijkmans, and Paul P. Tak

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Differential effects of leflunomide and methotrexate on cytokine production in rheumatoid arthritis.

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Keywords: leflunomide, PBMC, Interleukin-6, Interferon-gamma, methotrexate, proliferation
**Introduction.** T cells are considered to play a pivotal role in rheumatoid arthritis (RA) and, therefore, represent a potential target for treatment. The novel disease modifying anti-rheumatic drug (DMARD) leflunomide inhibits pyrimidine biosynthesis, to which T cells are especially susceptible, potentially resulting in a different cytokine profile compared with methotrexate.

**Materials and methods.** Serum samples of 100 RA patients, treated with leflunomide (n=50) or methotrexate (n=50), were collected at baseline, after 16 weeks and after 1 year of treatment. Serum levels of interleukin-6 (IL-6), and interferon (IFN)-γ were determined by ELISA. In addition, peripheral blood mononuclear cells (PBMC) of healthy volunteers (n=6) and RA patients (n=3) were isolated and the effects of the active metabolite of leflunomide (A77-1726, 0-200 µM) on cell proliferation (3H-thymidine incorporation) as well as on IL-6 and IFN-γ production were determined by ELISA. Furthermore, peripheral blood lymphocytes (PBL) and monocytes (PBM) were isolated from 2 healthy volunteers and we measured the effects of A77-1726 on IL-6 production by ELISA and PCR.

**Results.** Serum levels of IFN-γ were significantly reduced after leflunomide treatment (baseline 43 pg/ml ± 10 (mean ± s.e.m.); 1 year 29 ± 7 (P = 0.015), but there was no change in IL-6 levels (baseline 158 ± 41, 1 year 151 ± 48). In contrast, both IFN-γ and IL-6 levels were significantly reduced after methotrexate treatment. This observation was supported by in vitro experiments with A77-1726 displaying a reduced IFN-γ production in both healthy volunteers and RA patients without an effect on IL-6 production. The production of IFN-γ by PBL was inhibited by A77-1726, but IL-6 production by PBM was not inhibited.

**Conclusion.** The differential effect on IFN-γ and IL-6 production supports the hypothesis that activated T cells are preferentially inhibited by leflunomide. An explanation could be either inhibition of uridine synthesis or effects on signal transduction pathways.
Introduction.

Leflunomide inhibits both synovial inflammation and joint destruction in patients with rheumatoid arthritis (RA) (1-3). In vivo, leflunomide acts as a pro-drug and is quickly metabolised into the active metabolite A77-1726 in the gut wall and liver. Most of the in vitro pharmacodynamic studies have, therefore, been conducted with the active metabolite A77-1726 rather than with leflunomide.

The mechanism of action has been described in 3 excellent reviews (4-6). In summary, at least two modes of action of leflunomide have been documented: inhibition of dihydroorotate dehydrogenase (DHODH), by which leflunomide influences the de-novo pyrimidine biosynthesis (7), and interaction with primary and secondary signalling events (8-10).

The main target of leflunomide appears to be the pyrimidine biosynthesis, since there is a very high affinity binding to DHODH with low concentrations necessary to inhibit this enzyme (6). DHODH is essential for the de novo synthesis of uridine mono-phosphate (UMP), a precursor of pyrimidine nucleotides. Resting lymphocytes have low levels of DHODH and mainly use a salvage pathway for UMP to sustain survival (7). Activation of lymphocytes gives a 7-8 fold increased demand for UMP, which makes these cells susceptible to DHODH inhibition by leflunomide in the absence of a salvage pathway (11). DHODH inhibition results in decreased UMP levels, decreased DNA and RNA synthesis, and consequently inhibition of cell proliferation and G1 phase cell cycle arrest. Other cells are less affected by DHODH because of the use of a salvage pathway. Another argument supporting the proposed inhibitory effects of leflunomide on T cells by DHODH inhibition is the reversal of the observed effects by exogenous uridine in vitro (7). Further support is found in the observation that the inhibition of de-novo pyrimidine biosynthesis by leflunomide is 100 fold stronger than its effects on tyrosine kinases (6).

Leflunomide also has effects on signal transduction (9), interferes with cell-cell contact (12) and inhibits TNF-α induced activation of NF-κb (10). Moreover, studies on the effects of leflunomide have shown effects on neutrophil chemotaxis, which cannot directly be explained by effects on purine nucleotides (13). Therefore, it has been suggested that the effects on pyrimidine biosynthesis are associated with low doses leflunomide, whereas other mechanisms might be operative at higher concentrations (4;6).

In registration studies on the treatment of active RA the comparator drug for leflunomide was often methotrexate. The mechanism of action of methotrexate in RA is currently not completely understood but appears to be more than an effect on the purine biosynthesis, and appears to be not cell type specific. Where the effects of methotrexate on IL-6 (14) and IFN-γ (15) levels have previously been demonstrated, there are no data on leflunomide. To provide insight into the beneficial effects of leflunomide in patients with RA we examined the effects of leflunomide on cytokine production by mononuclear cells.

Materials and methods

In vivo study. A total of 100 (50 treated with leflunomide and 50 treated with methotrexate) patients were selected out of 999 RA patients who participated in a prospective, double blind, randomised clinical trial comparing leflunomide and methotrexate (16). First, sites with large number of patients enrolled were selected to minimize variance because of issues such as sampling, processing and sending to a centralized lab. Samples were stored at -80 °C and shipped to our centre for analysis 4 to 5 years after sampling. No date are available on stability but all samples were subject to the same conditions. As a result the serum samples of 6 study sites
were used. Of all 100 patients (50 leflunomide and 50 methotrexate) serum samples from baseline, after 4 months, and after 1 year were available and tested. Clinical variables included the disease activity score (DAS) and C-reactive protein (CRP), measured at identical time points. Patients were treated with either leflunomide 20 mg/day after a loading dose of 100 mg/day for the first 3 days or methotrexate 15 mg/week (initial dose 7.5 mg/week, increase to 10 mg/week 4 weeks after baseline, and increase to 15 mg/week 8 weeks after baseline). At baseline all DMARDs were washed out and only concomitant NSAID and low dose prednisone (10 ≤ mg/day) therapy was allowed.

Measurement of cytokine production by RA patients. The in vivo production of IL-6 was measured by ELISA using a monoclonal anti-human IL-6 antibody (R&D cat nr: MAB206), IFN-γ was measured by ELISA using a monoclonal anti-human IFN-γ antibody (R&D cat nr: DIF50). For both assays the manufacturer’s specifications were used.

In vitro experiments. To investigate the observations made in vivo in more detail we tested the effects of methotrexate and A77-1726 on peripheral blood mononuclear cells (PBMC), peripheral blood lymphocytes (PBL) and peripheral blood monocytes (PBM) in vitro. A77-1726 (the active metabolite of HWA486) obtained in powder form (kindly provided by Dr. R.R. Bartlett, Aventis Pharma, Wiesbaden, FRG), was diluted at the appropriate concentrations (0.1 to 200 µM, clinical relevant concentration is ± 20µM).

Cells. PBMC of healthy volunteers (n=5) and RA patients (n=3) were prepared using Ficoll density centrifugation. No major differences were found and, therefore, pooled results are presented of experiments in duplicate. In 2 healthy volunteers the PBL and PBM fractions were further purified by counter-current centrifugation, the preparations contained more than 80% PBL or PBM and the viability was more than 95% as assessed by trypan-blue exclusion.

Stimulation. Cells were stimulated with phytohaemagglutinin (PHA; Murex Diagnostics Lim., Dartford, England; 5 µg/ml), lipopolysacharide (LPS, Sigma, St. Louis, USA; 5 µg/ml), anti-CD2 monoclonal antibody (mAb) (CLB-CD2; 5 µg/ml), anti-CD3 mAb Okt3 (CLB-CD3, CLB, Amsterdam, The Netherlands; 5 µg/ml), and anti-CD28 mAb 15E8 (CLB-CD28/1; 5 µg/ml). The various stimuli were added to the PBMC, PBL, or PBM together with A77-1726 and intervals between 4 and 72 hours were tested.

Proliferation experiments. Overnight, 96-wells culture plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with anti-CD3 mAb or LPS. PBMC and PBL (2 donors) were plated (2 x 10^5 cells/well) and incubated with or without A77-1726 for 48 hours (24 hours after initiation 20µ Ci 3H-thymidine/ml, 0.05 ml/well was added). Cells were harvested and the rate of DNA synthesis was measured. Experiments were performed in triplicate.

Cytokine measurement. Experiments were performed in 24-well tissue culture plates (1 x 10^6 cells/well). For the determination of IL-6 an ELISA was used with anti-human IL-6 mAb (mAb16, Dept. of Nephrology LUMC, Leiden, The Netherlands), sensitivity was 1 pg/ml. For the determination of IFN-γ an ELISA was used with anti-IFN-γ mAb (MD2, CLB), sensitivity was 0.2 ng/ml.

For all experiments cell vitality and cell death were estimated by trypan blue exclusion and a lactate dehydrogenase (LDH) test (Boehringer Mannheim Cytotoxicity kit, Cat.No. 1 644 793). In none of the experiments the obtained results could be attributed to the effects of cell death. Addition of uridine together with A77-1726 at incubation abolished all observed effects of A77-1726 alone.
RNA preparation, cDNA synthesis, semiquantitative polymerase chain reaction (PCR) on PBM. In PHA stimulated PBM RNA encoding IL-6 and β2-microglobulin was measured by PCR as described previously (17). In brief, total RNA was isolated with RNAzol (Cinna/Biotecx Laboratories Inc., Houston, TX), according to the manufacturer’s description. Total RNA (2 µg) was converted into first-strand cellular cDNA using oligo-dT primers (Gibco BRL, Breda, The Netherlands). Relative quantification of mRNA was based on the usage of synthetic DNA (st-DNA=pQA-1) that contains sequences that are complementary to the cytokine specific PCR primers used and result in an amplification of different length than the specific amplicon. Titration experiments were carried out with various amounts of st-DNA added to a fixed amount of cellular cDNA (cell-DNA). At the titration used the intensity of the internal standard product and the specific IL-6 PCR was equal, and the amount of IL-6 product could be estimated. β2-microglobulin mRNA was employed for standardization of the different RNA samples. The ratio between IL-6 mRNA and β2-microglobulin mRNA was used to assess the relative levels of the specific mRNA between the various samples. The PCR mix (final volume 40µl) consisted of 1µl of a 1:10 dilution of cell-c-DNA, 2.5 nM of each dNTP, 50 µM KCL, 10 µM Tris-HCL (pH 8.4), 2 µM MgCl2, 0.06% BSA, 0.87 U of DNA taq polymerase (Perkin Elmer, Gouda, The Netherlands) and 10 pmol of each specific sense and anti-sense primer (Isogen Bioscience, Maarssen, The Netherlands) β2-microglobulin sense primer 5’GCAGCAGCGAATGGAAAGTC 3’, β2-microglobulin antisense primer 5’GATCCTGCTTACATGGTCTCG 3’, TNFα sense primer 5’ACCCGCCTGTAGCCCATGTT 3’, TNFα antisense primer 5’AAAGTAGACTTGCCCAGACT 3’.

Statistical analysis. Wilcoxon signed rank for paired samples were used to test the changes from the baseline measurement. Student-t tests were used to compare the 2 treatment groups. Spearman test were used to test correlations between CRP and IL-6.

Results

Study patients. Demographic and clinical data are depicted in Table 1. All patients had very active disease at baseline, as measured by the disease activity score (DAS) of 6.9 ± 0.1 (mean ± standard error of the mean) for the leflunomide patients and 7.0 ± 0.1 for the methotrexate patients, with a significant reduction after 4 months, and after 1 year in both leflunomide and methotrexate treated patients (Table 1). The C-reactive protein (CRP) levels were significantly reduced in both leflunomide and methotrexate patients (Table 1), but in line with previous results (18), the CRP levels were significantly lower after 1 year of treatment with methotrexate (P = 0.014).
Table 1. Demographics, clinical data and cytokine measurements of the RA patients studied.

<table>
<thead>
<tr>
<th></th>
<th>Leflunomide</th>
<th>Methotrexate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>N = 50 62 (46-75)</td>
<td>N = 50 58 (22-74)</td>
</tr>
<tr>
<td><strong>Disease duration</strong></td>
<td>49 ± 6</td>
<td>42 ± 5</td>
</tr>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAS</td>
<td>6.9 ± 0.1</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td>CRP</td>
<td>5.3 ± 0.9</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>43 ± 10</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>158 ± 41</td>
<td>107 ± 23</td>
</tr>
<tr>
<td><strong>4 Months</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAS</td>
<td>5.6 ± 0.2</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>CRP</td>
<td>2.2 ± 0.5</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>38 ± 8</td>
<td>44 ± 7</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>180 ± 67</td>
<td>107 ± 23</td>
</tr>
<tr>
<td><strong>1 Year</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAS</td>
<td>5.2 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>CRP</td>
<td>2.1 ± 0.5</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>29 ± 7</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>151 ± 48</td>
<td>44 ± 7</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.015</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.001</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* Data represent mean (Range), all other data represent mean ± standard error of the mean.

**In vivo cytokine measurements.** Leflunomide significantly inhibited IFN-γ production: baseline 43 ± 10 pg/ml (mean ± standard error of the mean (sem)), 1 year 29 ± 7 pg/ml (P = 0.015), whereas IL-6 remained unchanged (baseline 158 ± 41 pg/ml, 1 year 151 ± 48 pg/ml) (Table 1). Methotrexate also significantly inhibited IFN-γ levels: baseline 57 ± 10 pg/ml to 36 ± 6 pg/ml after 1 year (P = 0.046), but in contrast to the findings in the leflunomide treated patients IL-6 was also inhibited significantly at 4 months and after 1 year: baseline 107 ± 23 pg/ml, 4 months 93 ± 25 pg/ml (P = 0.020), and 1 year 80 ± 20 pg/ml (P = 0.05) (Table 1).

At baseline there was a significant correlation between serum IL-6 levels and serum CRP (rho 0.296, P = 0.003); this correlation was sustained at 4 months of treatment for methotrexate patients (rho 0.579, P < 0.0001), but not for leflunomide (rho 0.274, not significant). After 1 year there was no significant correlation between CRP levels and IL-6 in either leflunomide or methotrexate treated patients.

**In vitro studies.** A77-1726 completely inhibited mitogen induced PBMC proliferation by LPS and PHA (PHA and RA patients, control; 59,650 ± 8,835 counts (mean ± s.e.m.), 100 µM A77-1726; 1,744 ± 661 (P < 0.05, Figure 1 A) in a dose dependent fashion in both RA patients and healthy volunteers.

A77-1726 reduced IFN-γ production by PBMC after stimulation with PHA in a dose dependent way in healthy controls (control 18,549 ± 2772 pg/ml, 100µM A77-1726 6,810 ± 2,001 pg/ml (P < 0.05) (Table 2 and Figure 1 B) and RA patients (control; 4,484 ± 120 pg/ml, 100µM A77-1726 1,888 ± 32 pg/ml (P < 0.05). Stimulation with anti-CD3/anti-CD28 resulted in comparable results (Figure 1B). In PBL there was also a dose dependent inhibition (control 33,020 pg/ml, 100µM A77-1726 6,550 pg/ml). PBM were not tested for IFN-γ production.
Table 2. Interferon (IFN)-γ production (pg/ml) by peripheral blood mononuclear cells (PBMC) of rheumatoid arthritis (RA) patients and healthy controls at baseline and 48 hours after stimulation with PHA 5 µg/ml.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Controls</th>
<th>RA patients</th>
<th>Controls</th>
<th>RA Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>A77-1726 (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>18549 ± 2772</td>
<td>4484 ± 120</td>
<td></td>
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<tr>
<td>1</td>
<td>12 ± 36</td>
<td>0 ± 0</td>
<td>13500 ± 7267</td>
<td>4144 ± 12</td>
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<tr>
<td>10</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>15990 ± 6193</td>
<td>4474 ± 198</td>
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</tr>
<tr>
<td>25</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>10950 ± 6383</td>
<td>4106 ± 258</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>7830 ± 4848</td>
<td>3488 ± 320</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>6810 ± 2001</td>
<td>1888 ± 32</td>
<td></td>
</tr>
</tbody>
</table>

Data represent mean ± standard error of the mean. ND is not done

Consistent with the in vivo data we observed no effect of A77-1726 on production of IL-6 by PBMC after stimulation with PHA (control 13,967 ± 4,937 pg/ml (mean ± s.e.m.), 100 µM A77-1726 21,300 ± 4,198 pg/ml (Figure 1C). Stimulation with LPS resulted in comparable results (Figure 1C). In addition, A77-1726 did not affect IL-6 gene expression or protein production by PBM (baseline 24,306 ± 3,761 pg/ml, 100µM A77-1726  57,203 ± 18,221 pg/ml (Figure 2). As expected, IL-6 production by PBL was very low at baseline and did not change in the presence of A77-1726 (Table 3).
Table 3. Interleukin (IL)-6 production (pg/ml) by peripheral blood mononuclear cells (PBMC) of rheumatoid arthritis (RA) patients and healthy controls at baseline and 24 hours after stimulation with PHA 5 µg/ml. Also given are measurements in density centrifugation isolated peripheral blood monocytes (PBM) of healthy controls 8 hours after stimulation with PHA and peripheral blood lymphocytes (PBL) 16 hours after stimulation with αCD3/αCD28.

<table>
<thead>
<tr>
<th>Cells</th>
<th>PBMC</th>
<th>PBMC</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
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<tr>
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<td>PHA 8 hours Controls</td>
<td>αCD3/αCD28 16 hours Controls</td>
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</tr>
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<td>RA Patients</td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>3</td>
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<tr>
<td>A77-1726 (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>110 ± 11</td>
<td>13967 ± 4937</td>
<td>12880 ± 1640</td>
</tr>
<tr>
<td>1</td>
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<td>12033 ± 3134</td>
<td>10700 ± 680</td>
</tr>
<tr>
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<td>0 ± 0</td>
<td>0 ± 0</td>
<td>15067 ± 3162</td>
<td>10035 ± 815</td>
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<tr>
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<td>14600 ± 2872</td>
<td>11700 ± 1200</td>
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<tr>
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<td>198 ± 53</td>
<td>12633 ± 4513</td>
<td>16745 ± 4685</td>
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<tr>
<td>100</td>
<td>0 ± 0</td>
<td>133 ± 83</td>
<td>21300 ± 4198</td>
<td>16385 ± 725</td>
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</table>

Data represent mean ± standard error of the mean.
ND is not done

Discussion

We observed inhibition of IFN-γ but not of IL-6 in RA patients treated with leflunomide and, as previously described, inhibition of IL-6 (14) and IFN-γ (15) by methotrexate. Stimulation of PBMC, PBL and PBM confirmed the lack of effect on IL-6 production with a dose dependent inhibition of IFN-γ production and proliferation by A77-1726 (8;19-21).

For the understanding of the mechanism of action of new immunomodulating drugs, as applied for the treatment of RA, data on in vitro and in vivo cytokine production can disclose vital information on the targets of the investigated substance (22). As cytokines could reflect differential pharmacodynamical effects of leflunomide and methotrexate on inflammation we focused on the T cell related cytokine IFN-γ and the more macrophage related cytokine IL-6.

Serum IL-6 levels of RA patients have been associated with disease outcome (23), but failed to correlate with (24) disease activity in methotrexate treated RA patients, as demonstrated in our study. During phase III studies with leflunomide a remarkable observation was the significant clinical improvement with only modest changes in ESR and CRP, especially when compared to methotrexate. This relative lack of change in acute phase response after leflunomide treatment is consistent with a limited effect on IL-6 production and a more T cell-directed mechanism of action (25).

The T cell derived cytokine IFN-γ is also produced by Natural Killer cells (NK-cells) and is involved in nearly all phases of inflammation and in the regulation of inflammatory responses. It has effects on macrophage, B cell and neutrophil function. The inhibition of IFN-γ, as observed in this study, could be the result of inhibition of DODH impairing T-cell function with as secondary effect inhibition of monocyte/macrophage function. This is supported by the occurrence of inhibition at concentrations of active metabolite achieved in RA patients (1).The effects of leflunomide on IFN-γ production shown in the present study confirms and extends previous work.
in animal models of arthritis (26). Leflunomide has also been shown to interfere with IFN-γ induced iNOS activation and NO production in fibroblast (27), most likely through the MEK/MAP pathway (28). Unfortunately we were not able to establish a direct in vitro to in vivo comparison for methotrexate due to technical problems. However, the limited literature available suggest comparable results in an in vitro model as we observed in vivo (29;30) Within this context it is important to stipulate that the effects of methotrexate as a purine antagonist are likely limited (31) and that the beneficial effects are more likely mediated via adenosine (32-34). We observed a clear effect of leflunomide on the proliferation of PBMC without signs of cell death. Other authors have demonstrated that T cells are inhibited by leflunomide in the G1-S phase. Previously, these phenomena were attributed to an inhibitory effect on phosphorylation of tyrosine kinases with a result interference with signalling events. More recent data point strongly towards inhibition of the enzyme DHODH, resulting in a negative effect on pyrimidine biosynthesis and anti-proliferative effects. An overview of all data available suggests that the inhibitory effects of leflunomide are due to the combination of both inhibition of pyrimidine biosynthesis and interference with signalling events (6). The relative contribution of each mechanism of action might be dependent on the concentrations of the drug (26).

In conclusion, we observed a differential effect on cytokine production by leflunomide with a significant inhibition of IFN-γ production with unchanged IL-6 levels. This observation supports the hypothesis that leflunomide preferentially affects activated T cells. It also supports the clinical observation of different pharmacodynamical profiles of methotrexate and leflunomide (1).
This work was supported by a grant from Aventis Pharma.

**Figure 1.** (A) Incorporation of 3H-thymidine by PBMCs of healthy donors and RA patients after stimulation with LPS. Graph depicts the number of counts as measured after 24 hours of incubation in the presence of various concentrations of leflunomide (0-100 µM). (B) Production of IFN-γ (pg/ml) by PBMCs after 36 hours. Depicted are controls and cells stimulated with PHA in the presence of various concentrations of leflunomide (0-100 µM). (C) Production of IL-6 (pg/ml) by PBMCs after 8 hours. Depicted are controls and cells stimulated with PHA in the presence of various concentrations of leflunomide (0-100 µM).

**Figure 2.** Analysis of β2-M and IL-6 mRNA levels in PHA-stimulated monocytes. A constant volume of the β2-M and IL-6 cellular cDNA (cell-DNA) products was mixed with graded amounts of a known concentration of pQA1 DNA (st-DNA) containing the specific sequences for the β2-M and IL-6 PCR primers. PCR was performed, and the PCR products were separated by electrophoresis on a 1% agarose gel and visualized after ethidium bromide staining under UV transillumination. The concentration of st-DNA that gave an amount of PCR product equal to that of the cellular DNA β2-M or IL-6 PCR products, respectively, was determined. The intensity of the bands was quantified by densitometry. The density of the st-DNA was expressed as a percentage of the total density of st-DNA and cellular cDNA. Results are presented in the absence of A77-1726, and the presence of 10 µM and 100 µM of A77-1726.
Reference List


(12) Deage V, Burger D, Dayer JM. Exposure of T lymphocytes to leflunomide but not to dexamethasone favors the production by monocytes of interleukin-1 receptor antagonist and the tissue-inhibitor of metalloproteinases-1 over that of interleukin-1beta and metalloproteinases. Eur Cytokine Netw 1998;4;663-8.


A.
Proliferation (% Baseline)

B.
IFN-γ Pg/ml

C.
IL-6 (pg/ml)
Figure 2

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>β2-microglobulin</th>
<th>Interleukin-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM</td>
<td>A77-1726</td>
<td></td>
</tr>
<tr>
<td>10 µM</td>
<td>A77-1726</td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>A77-1726</td>
<td></td>
</tr>
</tbody>
</table>

Ratio IL-6 to β2 microglobulin

- IL-6 RNA
- β2 microglobulin-RNA
- IL6/β2-microglobulin