Dynamic effects of leflunomide on IL-1, IL-6, and TNF-α activity produced from peritoneal macrophages in adjuvant arthritis rats

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KEY WORDS leflunomide; interleukin-1; interleukin-6; tumor necrosis factor; macrophages; adjuvant arthritis

ABSTRACT

AIM: To investigate the effects of leflunomide (LEF) on modulating interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) production induced by lipopolysaccharide (LPS) in peritoneal macrophages (PMφ) in adjuvant arthritis rats and elucidate the possible mechanisms of antiinflammatory and antirheumatoid effects of LEF. METHODS: Freund’s complete adjuvant was injected in the hind footpad of rats to induce adjuvant arthritis (AA) rat model. The PMφ samples were taken at different time after medication. IL-1, IL-6, and TNF-α activities released from PMφ were measured by ELISA method or bioassay method. RESULTS: Production of IL-1, IL-6, and TNF-α was increased in the culture supernatant of PMφ in AA model rat. LEF could inhibit LPS-induced release of IL-1 and TNF-α from PMφ of the AA rats and the inhibitory effects were extremely rapid. LEF (10, 25 mg/kg) administrated for 21d could inhibit IL-6 release from PMφ in AA rats. CONCLUSION: The antiinflammatory mechanisms of LEF in AA rats might be related to inhibitory level of IL-1, IL-6, and TNF-α from PMφ in vivo.

INTRODUCTION

Rheumatoid arthritis (RA), an autoimmune disorder of unknown etiology, is characterized by chronic inflammation of synovial tissues and infiltration of the affected joints by blood-derived cells. RA is a disabling chronic disease characterized by autoimmune phenomena associated with multiple inflammatory mediators leading to structural damage to the joint[4].

Leflunomide (LEF) is a new disease-modifying antirheumatic drug (DMARD) of the isoxazol class. Following oral dosing, leflunomide is converted into an active metabolite, A77 1726. Leflunomide (Arava™) has recently been approved by the Food and Drug Administration of USA for the treatment of RA. LEF is a novel immunoregulatory and disease-modifying antirheumatic drug and it has been shown effective in treatment for RA in a number of clinical trials[2,3]. But the effects of LEF on activities level of cytokines,
interleukin-1 (IL-6), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) released from peritoneal macrophages (PMϕ) remain unknown. Considering the efficacy of leflunomide has been tested in animal models of RA conditions, we used the adjuvant arthritis (AA) in rats model as the RA model. The tests were designed to investigate the possible mechanism of anti-rheumatic effects of LEF.

MATERIALS AND METHODS

Reagents and drugs LEF (purity >99 %) was a white crystalloid powder obtained from College of Pharmacy, Second Military Medical University, Shanghai. Lipopolysaccharides (LPS, E coli 0111:B4), Concanavalin A (Con A), methyl thiazolyl tetrazolium (MTT), and RPMI-1640 medium were from Sigma Co. USA. Fetal calf serum (FCS) was purchased from Beijing Beijiao Farm Factory. Freund’s complete adjuvant (FCA) was from Sigma Co USA.

Rats and cell lines Wistar rat, ♀, weighing 150±20 g, from Animal Center of Health Science Center of Peking University. 7DT1 cells were provided by Department of Immunology, Health Science Center of Peking University.

Building AA rats model Each rat was injected in the hind footpad with FCA that contains 7 g/L of mycobacterium in a 0.1 mL volume.

Measurement of IL-1 activity AA rat model was built as above described; Exudated macrophages of rats were prepared by the intraperitoneal injection of 2.5 % glycogen solution. After 4 d, peritoneal exudated cells were collected with RPMI-1640 media. The cells were washed 3 times in RPMI-1640 media. The cell suspension was adjusted to 2×10⁹/L in RPMI-1640 medium containing 10 % FCS, and dispensed at 1 mL/well in 24-well plates. After 2 h incubation at 37 °C in 5 % CO₂, the nonadherent cells were removed by washing with RPMI-1640 medium. The cells were incubated with LPS (5 mg/L) from PMϕ, then the plates were incubated at 37 °C in a 5 % CO₂ atmosphere for 24 h. After incubation, the supernatant was collected by centrifugation (600×g, 10 min) and stored at −25 °C until assay.

IL-1 activity was evaluated by ELISA method.

Measurement of IL-6 activity IL-6 activity was determined utilizing the IL-6-dependent murine 7DT1 cells. Briefly, the cell density was adjusted to 1×10⁷/L and the cells was put into the well in 200 µL/well. The cells were cultured in the presence of test samples for 4 d and the IL-6 activity was measured utilizing the IL-6-dependent murine 7DT1 cells. The growth of 7DT1 cells was found to be dependent on IL-6. None of the other lymphokines was effective in supporting the growth of this clone. In fact, as little as 0.01 kU/L of IL-6 (equivalent to the activity exerted by rIL-6 2 ng/L) could specifically be detected using the 7DT1 cells. 7DT1 cells (1×10⁴/well, 200 µL) were cultured with various concentrations of test samples for 48 h and pulsed with MTT (5 mg/L, 10 µL/well) during the final 4 h.

Measurement of TNF-α activity AA rat model was built as above described; Exudated macrophages of rats were prepared by the intraperitoneal injection of 2.5 % glycogen solution. After 4 d, peritoneal exudated cells were collected. The cells suspended in RPMI-1640 containing 5 % heat-inactivated serum derived from normal rats were seeded to 24-well culture plates (1×10⁹/L) and incubated at 37 °C for 4 h. Nonadherent cells were removed by rinsing, and the remaining cells were used as the exudated macrophage preparation. The macrophages were cultured with LPS (5 mg/L, 1 mL) for 48 h. After the culturing, the TNF-α activity was determined by the ELISA assay.

Statistical analysis Data are expressed as the mean±SD of triplicate cultures. The statistical significance of the data was determined with Student’s t-test.

RESULTS

Dynamic effects of LEF on IL-1 level secreted from PMϕ in AA rats LEF was administrated from d 7 of building AA model. The drug was administrated for 21d, each day once time. The animals were sacrificed at d 14, d 21, and d 28. The level of IL-1 in supernatants increased significantly at 24 h after LPS 5 mg/L stimulation (Tab 1). LEF 25, 10, and 5 mg/kg inhibited the production of IL-1 from peritoneal mac-
Tab 1. Dynamic effects of LEF on IL-1 level secreted from PMφ in AA rats. *n=3 homogenates (each was pooled from 8 mice and assayed in triplicate). Mean±SD. *P<0.05 vs normal control. †P<0.05 vs AA control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>d 14</th>
<th>d 21</th>
<th>d 28</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Intracellular</td>
<td>Extracellular</td>
<td>Intracellular</td>
</tr>
<tr>
<td>Normal control</td>
<td>269±23</td>
<td>271±16</td>
<td>301±19</td>
</tr>
<tr>
<td>AA model control</td>
<td>377±7</td>
<td>383±16</td>
<td>336±6</td>
</tr>
<tr>
<td>LEF 5 mg/kg</td>
<td>324±3</td>
<td>256.0±0.0</td>
<td>325±40</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>307±48</td>
<td>267±16</td>
<td>270.0±1.0</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>282±9</td>
<td>249±10</td>
<td>282±13</td>
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Dynamic effects of LEF on IL-6 activity secreted from PMφ in AA rats The dynamic levels of IL-6 were assayed (Tab 2). The results showed that the production of IL-6 was not increased obviously in d 14. Until d 21, 28, the group of LEF 10 mg/kg and 25 mg/kg could inhibit LPS-induced release of IL-6. The results indicated that the appearance of inhibition effect on production of IL-6 was later than IL-1 and TNF-α. As the period administrated prolong, the IL-6 production was inhibited increasingly.

Dynamic effects of LEF on TNF-α level secreted from PMφ in AA rats From the Tab 3, the production of TNF-α increased obviously from d 14 till d 28 in AA model. LEF 10 mg/kg and 25 mg/kg could inhibit LPS-induced release of TNF-α. The results indicated that the inhibition effect on production of TNF-α from intracellular and extracellular were the same

Tab 2. Dynamic effects of LEF on IL-6 activity secreted from PMφ in AA rats. *n=3 homogenates (each was pooled from 8 mice and assayed in triplicate). Mean±SD. *P<0.05, †P<0.01 vs normal control. ‡P<0.01 vs AA control.

<table>
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<tr>
<th>Treatment</th>
<th>d 14</th>
<th>d 21</th>
<th>d 28</th>
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<tbody>
<tr>
<td></td>
<td>IL-6 activity (A)</td>
<td></td>
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<tr>
<td>Normal control</td>
<td>0.416±0.024</td>
<td>0.51±0.05</td>
<td>0.267±0.008</td>
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<tr>
<td>AA model control</td>
<td>0.50±0.04</td>
<td>0.766±0.010</td>
<td>1.36±0.20</td>
</tr>
<tr>
<td>LEF 5 mg/kg</td>
<td>0.46±0.03</td>
<td>0.69±0.08</td>
<td>0.33±0.12</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>0.45±0.07</td>
<td>0.67±0.04</td>
<td>0.32±0.12</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>0.43±0.03</td>
<td>0.617±0.018</td>
<td>0.22±0.03</td>
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DISCUSSION
In the present experiments, the AA rat model was used to investigate the possible mechanisms of anti-rheumatic effects of LEF. This model produced by sensitizing animals with sc injections of adjuvant. In this model, arthritis develops within 2 weeks and is characterized by reduced body weight, paw edema, and the development of inflammatory lesions. When given within 2 weeks of adjuvant injection, oral leflunomide inhibited the dermal hypersensitivity that characterized the initial stages of AA development[4].

IL-1 is a polypeptide produced mainly by macrophages, which has multiple biological activities including induction of the production of acute phase proteins by hepatocytes, and stimulation of prostaglandins and collagenase production by synovial cells[5]. On the basis of these facts, IL-1 is thought to be an essential mediator of inflammation. In particular, the importance of IL-1 in RA has been reported by various investigators[6]. For example, IL-1 production from RA synovium correlated not only with the degree of inflammation but also with that of joint destruction. Therefore, it is thought that an inhibitor of IL-1 generation could be a useful therapeutic agent in the treatment of RA. The results indicated that leflunomide could inhibit the IL-1 level secreted by PMϕ of the AA model rats and the inhibition effect on production of IL-1 was extremely rapid. It might be involved in its mechanisms of anti-inflammation and immunosupression. TNF-α and IL-6 are pro-inflammatory cytokines that play an important role in the pathogenesis of rheumatoid arthritis[7-9]. Both IL-1β and TNF-α can promote synovitis by direct effects on joint tissues and through induction of other proinflammatory cytokines. We could make a conclusion that the abnormal production of IL-1, IL-6, and TNF-α may be involved in the pathogenesis of autoimmune diseases and rheumatoid arthritis[10,11].

In conclusion, LEF showed inhibitory effects on the release of IL-1, IL-6, and TNF-α in AA model rats, which might be involved in mechanisms of its anti-inflammation and immunosuppresssive effects.

REFERENCES

坏死因子
巨噬细胞
佐剂性关节炎

目的
探讨来氟米特(leflunomide, LEF)对佐剂性关节炎大鼠腹腔巨噬细胞IL-1, IL-6和TNF-α分泌的影响及其抗炎抗类风湿的可能作用机制。

方法
大鼠足跖皮下注射Freund完全佐剂诱导关节炎模型，LEF灌胃后分次获取腹腔巨噬细胞，其培养上清液中IL-1, IL-6和TNF-α活性采用ELISA法或生物法测定。

结果
佐剂性关节炎大鼠腹腔巨噬细胞IL-1, IL-6和TNF-α分泌较正常对照组明显升高，LEF对由LPS诱导产生的IL-1和TNF-α有明显的抑制作用，作用产生快，LEF (10, 25 mg/kg)在应用21天后对IL-6的分泌也有明显抑制作用。

结论
来氟米特具有抑制佐剂性关节炎大鼠腹腔巨噬细胞IL-1, IL-6和TNF-α分泌水平的作用。