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World J Gastroenterol 2003;9(2):320-323
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

Effect of leflunomide on immunological liver injury in mice

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Received: 2002-07-31 **Accepted:** 2002-09-12

Abstract

AIM: To study the effect of leflunomide on immunological liver injury (ILI) in mice.

METHODS: ILI was induced by tail vein injection of 2.5 mg *Bacillus Calmette-Guerin* (BCG), and 10 d later with 10 μg lipopolysaccharide (LPS) in 0.2 mL saline (BCG+LPS). The alanine aminotransferase (ALT), aspartate aminotransferase (AST), nitric oxide (NO) level in plasma and molondiadehyde (MDA), glutathione peroxidase (GSHpx) in liver homogenate were assayed by spectroscopy. The serum content of tumor necrosis factors-α (TNF-α) was determined by ELISA. Interleukin-1 (IL-1), interleukin-2 (IL-2) and Concanavalin A (ConA)-induced splenocyte proliferation response were determined by methods of ^{3}H-infiltrated cell proliferation.

RESULTS: Leflunomide (4, 12, 36 mg· kg^{-1}) was found to significantly decrease the serum transaminase (ALT, AST) activity and MDA content in liver homogenate, and improve reduced GSHpx level of liver homogenate. Leflunomide (4, 12, 36 mg· kg^{-1}) significantly lowered TNF-α and NO level in serum, and IL-1 produced by intraperitoneal macrophages (PM_Φ). Moreover, the decreased IL-2 production and ConA-induced splenocyte proliferation response were further inhibited.

CONCLUSION: These findings suggested that leflunomide had significant protective action on ILI in mice.

Yao HW, Li J, Jin Y, Zhang YF, Li CY, Xu SY. Effect of leflunomide on immunological liver injury in mice. *World J Gastroenterol* 2003; 9(2): 320-323
http://www.wjgnet.com/1007-9327/9/320.htm

INTRODUCTION

Earlier studies have identified leflunomide, an isoxazole derivative, as a unique immunomodulatory agent capable of treating rheumatoid arthritis, allograft and xenograft rejection, systemic lupus erythematosus, prostate carcinoma, and neuronal-glial tumours, etc[1-12]. Our studies indicated that leflunomide had significantly therapeutic effects on the secondary inflammation response of adjuvant arthritis (AA) in rats. Recent evidence suggested the anti-inflammatory and immunoregulatory effects of leflunomide were related to its ability to suppress IL-1 and TNF-α selectively over their inhibitors in T lymphocyte/monocyte activation, and the activation of nuclear factor kappa B, a potent mediator of inflammation when stimulated by inflammatory egents[13-16]. Jankovic reported that A_{771726}, leflunomide' s active metabolite, also had inhibitory effect on NO production and iNOS mRNA expression in IFN-γ+LPS-activated murine and rat primary fibroblast[17, 18].

As we known, the activity of cytokines such as TNF-α, IL-1, IL-6, NO and T cell mediated immunity were closely related to the degree of liver injury caused by virus, endotoxin, ConA, and GalN[19-21]. Thus, inhibition of proinflammatory cytokines and regulation of host immunity would be beneficial to alleviating liver injury.

Based on the immunological dysfunction in liver injury and leflunomide' s immunomodulatory feature with high effication and low toxicity, we assumed that leflunomide might have therapeutic effect on ILI. To the best of our knowledge, however, there has been no report so far concerning the effect of leflunomide on ILI. In this study, therefore, we have clarified the therapeutical effect of leflunomide on ILI in mice.

MATERIALS AND METHODS

Animals and reagents

Male Kunming strain mice weighing 18-22 g were purchased from Animal Center of Anhui Medical University. Mice were allowed to take food and tap water *ad libitum*. Leflunomide was kindly donated by Cinkate Co., USA. ConA and LPS from *Escherichia coli* were purchased from Sigma Co., St. Louis, M, USA. 1, 1, 3, 1-tetraethoxypropane (TEP) and 5, 5' -dithibis-(2-nitrobenzicacid) (DTNB) were purchased from FLUKA Co., Switzerland. BCG was purchased from Institute of Shanghai Biological Products.

Preparation of ILI [22]

Each mouse was injected with 2.5 mg BCG (viable bacilli) in 0.2 mL saline via tail vein, and 10 d later with 10 μg LPS in 0.2 mL saline. At 0, 4, 8, and 12 h post-injection of LPS, animals received either leflunomide (4, 12, and 36 mg/kg, ig) or appropriate volume (25 mL/kg, ig) of vehicle (3 % prednisone). The mice were anesthetized with ether, then sacrificed by cervical dislocation 16 h after LPS injection and trunk blood was collected into heparinised tubes (50 U/mL) and centrifuged (1 500×g, 10 min, room temperature). Plasma was aspirated and stored at -70 ℃ until assayed as described below. The liver was also removed and stored at -70 ℃ until required.

Measurement of plasma ALT, AST, NO and TNF-α

Plasma ALT, and AST were determined using commercial kits produced by Institute of Shanghai Biological Products affiliated to the Ministry of Health. These activities are expressed as an international unit (U/L). Serum TNF-α and NO were measured using commercial kits produced by Sigma Co. and Beijing Biotinge-Tech., Co.Ltd, and their levels were expressed as pg· mL^{-1} and μmol· L^{-1} respectively.

Measurement of MDA and GSHpx in liver homogenate

Livers were thawed, weighed and homogenized with Tris-Hcl

buffer (5 mM containing 2 mM EDTA, pH 7.4). Homogenates were centrifuged (1 000×g, 10 min, room temperature) and the supernatant was used immediately for the assays of MDA and GSHpx. MDA was measured by the thiobarbituric acid method according to standard techniques (Gavino VG., 1981). The content of MDA was expressed as nmol per gram liver tissue. GSHpx was measured by the DTNB method, and its content was expressed as U per milligram protein.

Measurement of ConA induced splenocyte proliferation, IL-1 and IL-2

ConA induced splenocyte proliferation was determined according to the report by Yamamoto I in 1982. IL-1 and IL-2 were measured according to the reference (Liang JS, 1989; Ding GF, 1988).

Statistical analysis

Results were expressed by $\bar{x}\pm s$. Statistical significance of differences between groups were determined by ANOVA followed by Student' s *t* test. *P* value of less than 0.05 was considered statistically significance.

RESULTS

Therapeutic effects of leflunomide on ILI induced by BCG+LPS in mice

Results are shown in Tables 1 and 2. ALT, AST, and NO in plasma and MDA content in liver homogenate were significantly increased after the interval injection of BCG and LPS. Meanwhile, the GSHpx level in liver homogenate was sharply decreased. Both leflunomide (12, 36 mg/kg) and prednisone (3 mg/kg) could not only significantly decrease ALT, AST, NO and MDA level, but evidently increase GSHpx in mice with ILI.

Table 1 Effects of leflunomide on serum ALT and AST activities induced by BCG+LPS in mice (n=10, $\bar{x}\pm s$)

Groups	Dose (mg·kg^{-1})	ALT (u·L^{-1})	AST (u·L^{-1})
Normal		32.1±5.6	35.8±6.4
Model		195.4±21.8^{d}	188.4±22.5^{d}
Leflunomide	4	181.5±19.5^{d}	175.2±18.1^{d}
	12	173.8±15.8^{ad}	166.5±15.7^{ad}
	36	121.8±11.5^{bd}	108.2±9.8^{bd}
Prednisone	3	81.5±7.8^{bd}	64.7±5.8^{bd}

^{a}P<0.05, ^{b}P<0.01 *vs* model group; ^{d}P<0.01 *vs* normal group.

Table 2 Effects of leflunomide on serum NO, MDA and GSHpx contents in liver homogenates induced by BCG+LPS in mice (n=10, $x\pm s$)

Groups	Dose (mg·kg^{-1})	Plasma NO (μM)	Liver homogenates	
			MDA (nmol/g tissue)	GSHpx (μ/mg protein)
Normal		8.8±1.0	133.2±14.5	163.9±15.9
Model		74.5±10.1^{d}	395.9±23.6^{d}	62.5±8.8^{d}
Leflunomide	4	68.3±8.5^{d}	385.7±22.2^{d}	66.3±9.1^{d}
	12	60.7±7.1^{bd}	363.9±19.3^{bd}	87.1±9.9^{bd}
	36	55.3±6.2^{bd}	301.9±17.1^{bd}	95.1±10.7^{bd}
Prednisone	3	44.4±5.3^{bd}	272.0±15.7^{bd}	108.0±12.0^{bd}

^{a}P<0.05, ^{b}P<0.01 *vs* model group; ^{d}P<0.01 *vs* normal group.

Effects of leflunomide on TNF-α

As shown in Table 3, when the mice were first injected with BCG and then challenged with LPS, the level of TNF-α was elevated significantly. Leflunomide (4, 12, and 36 mg/kg) obviously decreased the increased TNF-α level in serum.

Table 3 Influences of leflunomide on serum TNF-α induced by BCG+LPS in mice (n=8, $\bar{x}\pm s$)

Groups	Dose (mg·kg^{-1})	TNF-α (pg·mL^{-1})
Normal	-	Under detection limit
Model	-	353.3±28.7^{d}
Leflunomide	4	305.0±31.4^{ad}
	12	240.0±31.1^{bd}
	36	140.0±31.1^{bd}
Prednisone	3	88.7±25.6^{bd}

^{a}P<0.05, ^{b}P<0.01 *vs* model group; ^{d}P<0.01 *vs* normal group.

Influence of leflunomide on IL-1

IL-1 excreted by PM_{Φ} was significantly increased in the model group. As shown in Table 4, Leflunomide (4, 12, and 36 mg/kg) evidently inhibited PM_{Φ} excreting too much IL-1.

Table 4 Influences of leflunomide *in vivo* on IL-1 and IL-2 production and splenocyte proliferation in mice induced by BCG+LPS. (unit:10^{3}cpm) (n=8, $\bar{x}\pm s$)

Groups	Dose (mg·kg^{-1})	IL-1	IL-2	Splenocyte proliferation
Normal		11.2±2.40	13.3±1.76	17.5±2.26
Model		34.6±3.96^{d}	9.3±1.57^{d}	7.9±1.19^{d}
Leflunomide	4	29.6±3.71^{ad}	8.2±1.44^{d}	7.0±1.01^{d}
	12	18.9±3.28^{bd}	7.6±1.31^{ad}	6.4±0.95^{ad}
	36	16.6±3.08^{bd}	6.5±1.20^{bd}	5.2±0.87^{bd}
Prednisone	3	15.7±2.85^{bd}	5.0±1.12^{bd}	4.4±0.71^{bd}

^{a}P<0.05, ^{b}P<0.01 *vs* model group; ^{d}P<0.01 *vs* normal group.

Effect of leflunomide on IL-2 generation and ConA induced splenocyte proliferation

IL-2 and ConA induced splenocyte proliferation were significantly inhibited in the model group (Table 4). Leflunomide (4, 12, and 36 mg/kg) further inhibited IL-2 production and ConA induced splenocyte proliferation response.

DISCUSSION

It has been demonstrated that severe hepatitis could be induced by injecting a small dose of bacterial LPS into BCG-pretreated mice[22]. In this article, ILI was successfully induced by BCG+LPS. On this basis, leflunomide (4, 12, and 36 mg/kg) could significantly lower the increased plasma transaminase level and MDA content in liver homogenate, meanwhile, GSHpx level rose significantly. All these indicated that leflunomide markedly protected ILI. Leflunomide significantly inhibited the generation of NO, TNF-α and IL-1 excreted by PM_{Φ}, moreover, IL-2 production and ConA induced splenocyte proliferation was further inhibited by leflunomide. Therefore, the protective effects of leflunomide on ILI might be related with its function of balancing cytokine generation and modulating immune.

As it is known, TNF-α is one of the important mediators in liver injury. It has been demonstrated that liver injury induced by endotoxin was conducted by TNF-α, and the

activity of TNF-α was positively related with the extent of liver necrosis[22-24]. However, TNF-α itself could not directly result in liver injury. The damaging degree of TNF-α on liver might be involved with infection, activity of Kupffer cell, and endogenous serine type protease, etc[25-30]. TNF-α could act as the first factor of liver injury, its elevation would stimulate a number of proinflammatory mediators including NO, IL-1, IL-6, IL-8 and SIL-2R[31-36], which further deteriorated the liver injury intoxicated by TNF. Therefore, although the TNF lever was low, liver was damaged significantly.

Leflunomide, an immunomodulatory reagent, is mainly aimed to inhibit the activity of dihydroorotate dehydrogenase (DHODH) involved in *de novo* pyrimidine biosynthesis. But at a higher concentration, it mainly inhibited protein tyrosine kinases initiating signaling[1,13,14,37,38], and therefore could reduce the cell response to mitogen and cytokine. In the model of ILI induced by BCG+LPS, leflunomide could significantly lower the increased TNF-α level in serum, which agreed with the results of Smith' s experiment that leflunomide significantly lowered the increased TNF level in joints from AA rats[15,16,39]. As it is known, TNF mainly come from Kupffer cell in liver. In this article, leflunomide significantly inhibited TNF-α level in serum of ILI. It deserved further investigation on about whether it is related to leflunomide' s effect of regulating the immunological dysfunction through inhibiting the growth and differentiation of Kupffer cell and production of TNF, thus, alleviating liver injury.

As reported in documents, the synthesis of NO was regulated by many immunological factors including TNF-α, IL-1, and IFN-γ, which is composed of a complicated web system, could act on hepatocytes, Kupffer cells and Ito in endotoxemia mice to increase the generation of NO[31,32,35,40]. Likewise, LPS could also induce Ito cells to express iNOS and synthesis of a large amount of NO[41,42]. According to our investigation, the effects of leflunomide to inhibit ILI might well be related with its function of decreasing the degeneration of NO.

Although IL-1 itself has no damage on liver, its elevation could stimulate many kinds of immunological and inflammatory cells to excrete cytokine including TNF-α, IFN-γ, IL-6, and IL-8, which mediate the inflammatory and immunological injury. Apart from these, IL-1,TNF-α, IFN-γ and LPS could act on hepatocyte to enhance the expression of iNOS mRNA in synergetic manner, and to increase the generation of NO, thus deteriorating the liver injury. Leflunomide significantly regulated abnormal IL-1 level excreted by PM_{Φ} in ILI mice *in vivo*, which agrees with Deage' s investigation[43] in effect of leflunomide on AA rats.

Suzuki found that splenectomy could modulate the excretion of inflammatory mediators, which prevented liver injury intoxicated by LPS after hepatectomy. In this study, we discovered that IL-2 production and ConA induced splenocyte proliferation were reduced in ILI induced by BCG+LPS. However, leflunomide further inhibited the production of IL-2 and ConA induced splenocyte proliferation response. Hoskin *et al*[44] reported that leflunomide inhibited the T lymphatic cell growth and response to IL-2 and production of IL-2. Further studies are needed to elucidate the relationship between the protective effect of leflunomide on ILI and its inhibitory action on cellular immune function.

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Edited by Ma JY