BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1998 May; 19 (3): 257 - 260

Cytokine and nitric oxide production by rat microglia stimulated with lipopolysaccharides *in vitro*

DU Ze-Ying, LI Xiao-Yu[1]
(*Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS interleukin-1; interleukin-2; tumor necrosis factor; nitric oxide; microglia; lipopolysaccharides; cultured cells

AIM: To study the characterization of interleukin (IL)-1, IL-2, tumor necrosis factor-α (TNF-α), and nitric oxide (NO) production in microglia stimulated with lipopolysaccharides (LPS). **METHODS**: Primary cultured neonatal rat microglia were incubated with LPS (0 - 10 mg $\cdot L^{-1}$) for 0 - 72 h. The supernatants and lysates were collected. IL-1, IL-2, and TNF-α were assayed by mouse thymocyte proliferation, mouse spleen cell proliferation, and L929 cytotoxity, respectively. NO was assayed by Griess reaction. **RESULTS**: Extracellular IL-1, TNF-α, and NO production reached peak levels at LPS 1 mg$\cdot L^{-1}$. Intracellular IL-1 production reached its peak level at LPS 100 μg $\cdot L^{-1}$. Intracellular TNF-α level was very low. IL-1, TNF-α, and NO activities were detected at 1, 4, and 8 h, after the cells were stimulated with LPS. IL-1 got to its peak value at 8 h, TNF-α, and NO reached the highest levels at 24 h. However, IL-2 activity was not detected after the microglia were stimulated with LPS 0 - 10 mg $\cdot L^{-1}$ *during the incubation period*. **CONCLUSION**: Rat microglia stimulated with LPS *in vitro* produced proinflammatory cytokines and NO.

Microglia, derived from monocytes arising from the bone marrow early in fetal development, are the ontogenetic and functional equivalents of mononuclear phagocytes in a variety of tissues[1], eg, Kupffer cells in the liver and Langerhans cells in the skin. Microglia not only play a role in the development and differentiation of central nervous system (CNS)[2], but are also implicated in the pathogenesis of a number of neurodegenerative diseases, such as, Alzheimer's disease[3], AIDS dementia[4], and multiple sclerosis[5]. Besides their potential roles in host defense and tissue repair, microglia may also be destructive. It is suggested that proinflammatory cytokines, such as IL-1, IL-6, and TNF-α, produced by activated microglia are involved in microglia mediated neuronal damage. Nitric oxide (NO) is a molecule, having neurotransmission, vasodilatation, antimicrobial activity, and cytotoxicity. Cytokines and NO may participate in the process of CNS cell apoptosis, differentiation, and proliferation, as well as modulation of inflammation and immune responses. In this paper, highly purified microglia from neonatal rats were used to study the biological activities of IL-1, IL-2, TNF-α, and the level of NO after stimulation with LPS.

MATERIALS AND METHODS

Reagents Lipopolysaccharides (*E Coli* O55 : B5), concanavalin A (Con A), glial fibrillary acidic protein (GFAP) monoclonal antibody (MAb), and goat anti-mouse IgG peroxidase conjugate were purchased from Sigma. ED1 MAb was a gift from Dr C D Dijkstra (Department of Cell Biology and Immunology, Faculty of Medicine, Free University, Amsterdam, The Netherlands). Dulbecco's modified Eagle's medium (DMEM, high glucose, Gibco BRL) was supplemented with 10 % newborn bovine serum (Gibco BRL), *L*-glutamine 2 mmol $\cdot L^{-1}$, sodium pyruvate 1 mmol $\cdot L^{-1}$, benzylpenicillin 100 kU$\cdot L^{-1}$ and streptomycin 100 mg $\cdot L^{-1}$. RPMI-1640 medium (Gibco BRL) was supplemented with 10 % newborn bovine serum, *L*-glutamine 2 mmol$\cdot L^{-1}$, sodium pyruvate 1 mmol$\cdot L^{-1}$, HEPES 10 mmol$\cdot L^{-1}$, 2-mercaptoethanol 50 μmol$\cdot L^{-1}$, benzylpenicillin 100 kU$\cdot L^{-1}$, and streptomycin 100 mg$\cdot L^{-1}$. [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium)] (MTT) was from Fluka Co. Sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDA), and phosphoric acid were domestic AR grade products. Griess reagent was composed of 1 % sulfanilamide and

[1] Correspondence to Prof LI Xiao-Yu. Phn 8-21-6431-1833, ext 317. Fax 86-21-6437-0269. E-mail xyli@server.shcnc.ac.cn
Received 1997-07-18 Accepted 1997-12-25

0.1 % NEDA in 4 % phosphoric acid.

Mixed glial culture Mixed glial cultures were prepared from cerebra of neonatal Sprague-Dawley rats (within 24 h) by modified method[6]. Meninges were carefully removed, brain tissues were minced and dissociated by gently pipetting in phosphated buffered saline (PBS), pH 7.4. Tissue suspensions were then passed through 120 and 80 μm diameter stainless steel meshes successively. Cells were seeded at glass tissue culture flasks pre-coated with collagen from rat tail in 5×10^9 cells · L^{-1} and incubated at 36.5 ℃ in a humidified air with 5 % CO_2. At time of primary cell confluence (d 14), microglia were harvested by gentle shaking flasks for 3 – 4 h on horizontal shaking platform (LAB-LINE/DINOFF SHAKER). At the every subsequent week, microglia can be harvested from the same astrocyte (feeder) cultures[7].

Microglial cultures The supernatants were collected and centrifuged at 200 × *g* for 5 min. The pellets were washed twice. Cells resuspended in DMEM were seeded in 48-well plastic plates (Costar) at 2×10^8 cells · L^{-1} for adherence for 30 min. Cultural media were removed. After the cells attached, they were supplied with fresh media and continuously cultured overnight to allow them recover from mechanical shaking and washing. The cells were evaluated[8] by histochemical marker non-specific estatase staining 99 % as positive, and immunocytochemical microglial marker ED1 staining more than 95 % and astroglial marker GFAP staining less than 5 %.

Induction of cytokine and NO production by microglial culture The original medium of primary rat microglia in 48-well plates (Costar) was aspirated off and replaced with 0.5 mL fresh medium containing LPS 0 – 10 mg · L^{-1} and continuously cultured for 72 h. Every assay was done in duplicate. The supernatants were collected. The residues were washed 3 times with PBS and frozen-thawed for 3 cycles. Culture media 0.5 mL were added to each well and vetexed for 1 min. The plates were centrifuged at 500 × *g* for 10 min and the supernatants were collected. Both of the supernatants were stored at – 20 ℃ until assay.

Assay for IL-1 activity IL-1 activity was performed by modified thymocyte proliferation assay[9]. Thymocytes (2×10^6 cells/well) of BALB/c mice were seeded in 96-well plates in RPMI-1640. Serial dilutions of the samples or of rIL-1 standard and Con A (final concentration was 3 mg · L^{-1}) were added to the wells. After 68-h incubation at 36.5 ℃ in 5 % CO_2, MTT 20 μL (5 g · L^{-1} in PBS) was added and incubated for 4 h. The plates were centrifuged at 1000 × *g* for 10 min. After aspiration of the medium, 100 μL of Me_2SO was added to dissolve the formazan particles and the absorbance at 570 nm was measured with an ELISA reader.

Assay for TNF-α activity Biological activity was evaluated by using cytotoxicity assay against L929 cells[10].

Assay for IL-2 activity Biological activity of IL-2 was determined by modified activated spleen cell proliferation assay[11]. The Con A-primed mouse spleen cells (2×10^6 cells/well) and diluted samples or human rIL-2 standard were incubated at 36.5 ℃ in 5 % CO_2. MTT 20 μL (5 g · L^{-1} in PBS) was added and the next procedures were as these of IL-1 assay.

Assay for NO production[12] The supernatants were mixed with 100 μL of Griess reagent. After chromophore was formed at 25 ℃ for 15 min, absorbance was determined at 570 nm with an ELISA reader. NO levels were represented as nitrite concentration, and were determined with reference to a standard curve of sodium nitrite.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed by *t*-test.

RESULTS

IL-1 production Rat microglia began to produce IL-1 after LPS 1 mg · L^{-1} stimulation for 1 h. IL-1 reached the peak level at 8 h and sustained a relatively high level until 24 h. There was a similar character between the supernatants (extracellular IL-1) and lysates (intracellular IL-1). IL-1 was initially produced at LPS 1 μg · L^{-1}, and LPS 0.1 and 1 mg · L^{-1} stimulated the intra- and extra-cellular highest level, respectively (Fig 1).

TNF-α production The production of TNF-α was later than that of IL-1. It began to release at 4 h and got to the peak level at 24 h. LPS 1 μg · L^{-1} stimulated TNF-α production and 1 mg · L^{-1} induced the highest TNF-α levels (Fig

1). But little amount of TNF-α were detected in the lysates.

NO production Rat microglia stimulated with LPS 1 mg·L^{-1} produced NO at 8 h. NO reached the peak level at 24 h and sustained relatively high levels at 48, and 72 h (Fig 1). LPS 1 μg·L^{-1} stimulated NO production and 1 mg·L^{-1} produced the highest level of NO (Fig 1).

IL-2 production Rat microglia stimulated with LPS 0 – 10 mg·L^{-1} did not produce IL-2 during 72-h incubation whatever in extracellular or intracellular supernatants.

DISCUSSION

This study showed that rat microglia produced high amount of IL-1, TNF-α and NO in LPS-stimulated cultures. The results showed that IL-1 production was the earliest event, then TNF-α and NO which formed the inflammatory

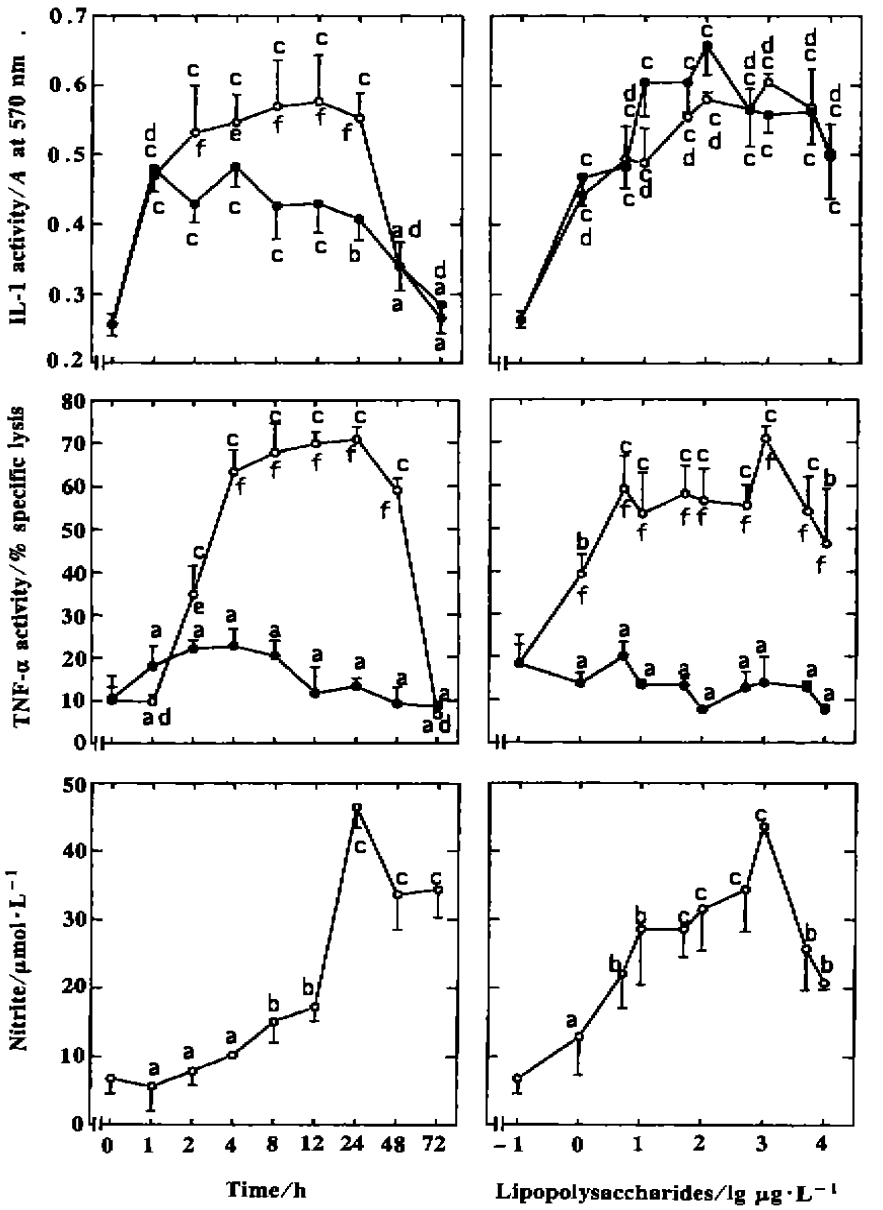

Fig 1. Extracellular (○) and intracellular (●) IL-1, TNF-α, and nitrite production by rat microglia stimulated with LPS *in vitro*. $n = 3$ experiments (2×10^5 cells·L^{-1} from 12 – 14 neonatal rats, 0.5 mL/well), $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* intracellular.

cascades leading to tissue injuries. It was suggested that the initial response to stimuli in the CNS be elicited by microglia and then lead to response in astrocytes, primary through the production of IL-1[13].

Our results showed that microglia were activated at very low concentration of LPS and at very early stage after LPS stimulation. It supports that microglia are responsible for establishing and maintaining the brain homeostatic environment because they are activated at a very early stage of injury and in response to even minor pathological changes[14]. Moreover, microglia are the central cellular elements of senile plaques in Alzheimer's brains. The features of microglial activation observed in Alzheimer's disease, multiple sclerosis, and Parkinson disease are very similar. They may not only release growth factors, such as IL-1, to increase their cell number, but also produce substances harmful to adjacent neurons. The substances are excitatory amino acids, reactive oxygen intermediates, nitric oxide, and cytokines which are produced by cultured microglia exposed to plaque material from Alzheimer's brains. In addition, both IL-1 and TNF-α immunoreactivity have been found on activated microglia surrounding senile plaques. IL-1 released by activated microglia has been shown to induce astrocytic proliferation as well as to stimulate neovascularization in brain trauma. IL-1 inhibits oligodendrocyte proliferation and differentiation *in vitro*. TNF-α exerts a cytotoxic effect on myelinating oligodendrocytes *in vitro*. Many observations suggest that TNF-α is a key deleterious factor in the development and relapse of multiple sclerosis. Microglia are also a major source of NO synthase and NO. Amyloid protein, LPS, and interferon-γ stimulated microglia to produce NO which caused neuron injury and death[15]. Thus, the intervention of activated microglia may be a target of drugs in reducing CNS inflammatory, ischemic, and neurodegenerative diseases.

REFERENCES

1 Gehrmann J, Matsumoto Y, Kreutzberg GW. Microglia: intrinsic immuneffector cell of the brain. Brain Res Rev 1995; 20: 269 – 87.

2 Merrill JE. Tumor necrosis factor alpha, interleukin 1 and related cytokines in brain development: normal and pathological Dev Neurosci 1992; 14: 1 – 10.

3 Rogers J, Luber-Narod J, Styren SD, Civin WH. Expression of immune system-associated antigens by cells of the human central nervous system: relationship to the pathology of Alzheimer's disease. Neurobiol Aging 1988; 9: 339 – 49.

4 Merrill JE. Chen ISY. HIV-1, macrophages, glial cells, and cytokines in AIDS nervous system disease FASEB J 1991; 5: 2391 – 7.

5 Matsumoto Y, Ohmori K, Fujiwara M. Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under *in vivo*-mimicking conditions. Immunology 1992; 76: 209 – 16.

6 Keller M, Jackisch R, Seregi A, Hertting G. Comparison of prostanoid forming capacity of neuronal and astroglial cells in primary cultures. Neurochem Int 1985; 7: 655 – 65.

7 Appel K, Honegger P, Gebicke-Haerter PJ. Expression of interleukin-3 and tumor necrosis factor-β mRNAs in cultured microglia. J Neuroimmunol 1995; 60: 83 – 91

8 Hayes GM, Woodroofe MN, Cuzner ML. Characterization of microglia isolated from adult human and rat brain. J Neuroimmunol 1988; 19: 177 – 89.

9 Xiang DB, Li XY. Effects of *Achyranthes bidentata* polysaccharides on interleukin-1 and tumor necrosis factor-alpha production from mouse peritoneal macrophages. Acta Pharmacol Sin 1993; 14: 332 – 6.

10 Fliek DA, Gifford GE. Comparison of *in vitro* cell cytotoxic assays for tumor necrosis factor J Immunol Methods 1984; 68; 167 – 75.

11 Ding GF, Xi HL, Deng YL, Deng HY, Shang HS. Measurement of IL-2 by activating mouse spleen cells. Shanghai J Immunol 1988; 8: 64 – 7.

12 Kondo Y, Takano F, Hojo H. Inhibitory effect of bisbenzylisoquinolinealkaloids on nitric oxide production in activated macrophages. Biochem Pharmacol 1993; 46: 1887 – 92.

13 Lee SC, Liu W, Dickson DW, Brosnan CF. Cytokine production by human fetal microglia and astrocytes; differential induction by lipopolysaccharide and IL-1β. J Immunol 1993; 150; 2659-67.

14 Merrill JE, Benveniste EN. Cytokines in inflammatory brain lesions: helpful and harmful. Trend Neurosci 1996; 19: 331 – 8.

15 Meda L, Cassatella MA, Szendrei GI, Otvos L Jr, Baron P, Villalba M, *et al*. Activation of microglial cells by β-amyloid protein and interferon-γ. Nature 1995; 374: 647 – 50.

25)-26 0 (15

脂多糖刺激体外大鼠小胶质细胞产生细胞肽和一氧化氮

R977.6

杜泽英，李晓玉
（中国科学院上海药物研究所，上海 200031，中国）

关键词 白细胞介素-1；白细胞介素-2；肿瘤坏死因子；一氧化氮；小胶质细胞；脂多糖；培养的细胞

目的：研究 LPS 刺激体外培养的新生大鼠小胶质细胞产生 IL-1，IL-2，TNF-α 和 NO 的特征．**方法**：小胶质细胞与 LPS（0 – 10 mg·L^{-1}）孵育 0 – 72 h，分别测定细胞外和细胞内的 IL-1，IL-2 和 TNF-α 的生物活性和细胞外 NO 水平．**结果**：IL-1，TNF-α 和 NO 分别在 LPS 刺激后 1，4，和 8 h 检测到，并在 8，24 和 24 h 达到峰值．LPS 1 mg·L^{-1}刺激细胞外 IL-1，TNF-α 和 NO 的产生最高，但细胞内 TNF-α 水平极低，LPS 未能刺激 IL-2 产生．**结论**：体外 LPS 刺激大鼠小胶质细胞产生大量炎性细胞肽和 NO．