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Inhibitory effects of triazolodiazepine on mouse splenocytes and peritoneal macrophages *in vitro*

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ABSTRACT Effects of triazolodiazepine (Tri) on mouse splenocyte proliferation and macrophage phagocytosis were studied. Tri 0.1—10 $\mu mol \cdot L^{-1}$ significantly inhibited concanavalin A (Con A) 5 $\mu g \cdot ml^{-1}$ and lipopolysaccharides (LPS) 10 $\mu g \cdot ml^{-1}$-induced splenocyte proliferation and phagocytic activity of activated peritoneal macrophages. Tri 10 $\mu mol \cdot L^{-1}$ reduced Con A induced colony stimulating factor release from mouse splenocytes and LPS-challenged intracellular and extracellular interleukin-1 production from peritoneal macrophages. These results partially explain the wide and strong anti-inflammatory effects of Tri.

KEY WORDS triazolodiazepine; interleukin-1; colony-stimulating factors; macrophages; phagocytosis; spleen

The participation of platelet-activating factor (PAF) in inflammatory and immunological responses has been studied intensively. PAF antagonists inhibited most of PAF effects *in vitro* and *in vivo*[1,2]. Triazolodiazepine (Tri, WEB 2086) is a hetrazepine PAF antagonist. Previous work emphasized on its antagonism of PAF effects and paid little attention to its effect on immune process[2]. We speculated that its strong and wide anti-inflammatory effects might be linked to its inhibition on immunological reactions. In the present study, we observed the effects of Tri on mitogen-induced mouse splenocyte proliferation and colony stimulating factor (CSF) secretion. Effects of Tri on phagocytic activity and interleukin-1 (IL-1) production of activated peritoneal macrophages were also studied.

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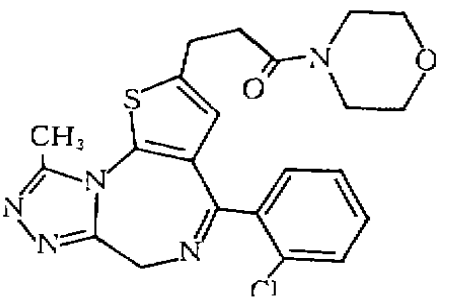

Triazolodiazepine (WEB 0286)

3-[4-(2-Chlorophenyl)-9-methyl-6*H*-thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone

$C_{22}H_{22}ClN_5O_2S$ $M_r = 455.97$

MATERIALS AND METHODS

Chemicals and mice Tri was gifted from Boehringer Ingelheim KG, Ingelheim am Rhein, Germany. Concanavalin A (Con A), lipopolysaccharides (LPS, *E coli* 055:B5), neutral red, and RPMI-1640 medium were purchased from Sigma Chemical Co, USA. [^{3}H]TdR (811 TBq $\cdot$ mol^{-1}) was obtained from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. Fetal calf serum (FCS) was purchased from Department of Pathology, Second Military Medical University. BALB/c and ICR mice of either sex weighting 16—24 g were supplied by Animal Center, Second Military Medical University.

Splenocyte proliferation BALB/c mice were killed by cervical dislocation and spleen cells were prepared[3]. Splenocytes at 5×10^5/well in 0.1 ml RPMI-1640 containing 10% heat-inactivated FCS were seeded into 96-well flat-bottom microtiter plates. Tri was added to the cells in the presence or absence of Con A 5 $\mu g \cdot ml^{-1}$ or LPS 10 $\mu g \cdot ml^{-1}$ and incubated in a humidified 5% CO_2 atmosphere at 37 °C. [^{3}H]TdR 1850 Bq/well was added for the last 12 h of culture period. Cells were harvested at 72 h and cell associated

radioactivity was measured in a FJ-2107 scintillation counter (Xi-an, China).

CSF assay Con A 5 $\mu g \cdot ml^{-1}$ alone or in combination with Tri were added to murine splenocytes at 5×10^6 cells per well per ml to induce CSF. After 96 h of incubation in a 5% CO_2 incubator at 37 C, supernatants were harvested by centrifugation ($1000 \times g$, 10 min). CSF content was assessed using mouse bone marrow cell (BMC) proliferation assay[5]. Briefly, BMC from ICR mice was seeded at 2×10^4 cells per well in 0.1 ml RPMI-1640 with 20% FCS and 20% horse serum. Another 0.1 ml of RPMI-1640 containing serial dilutions of test samples was added to each well. After 90 h of incubation the cells were pulsed with [^{3}H]TdR 1850 Bq per well for 6 h. The cells were harvested using a microplate cell harvester and the incorporation of [^{3}H]TdR was determined by scintillation counting.

IL-1 measurement Peritoneal exudate cells were obtained from thioglycollate-primed ICR mice[6]. The cells were added at 2×10^6 cells per well in 1 ml RPMI-1640 containing 10% FCS for 2 h in a humidified incubator at 37 C with 5% CO_2 to facilitate cell adherence. The media was discarded and LPS 10 $\mu g \cdot ml^{-1}$ were added to each well in a final volume of 1 ml RPMI-1640 alone or together with different concentrations of Tri to induce IL-1. After 24 h of culture the media were collected and centrifugated to get supernatants. One ml of fresh medium was added to the cell monolayer and the samples were frozen. After 3 cycles of freezing/thawing, the IL-1 activity in these samples was referred as the cell-associated one. IL-1 content in these samples and the supernatants was assessed using Con A (2.5 $\mu g \cdot ml^{-1}$)-induced ICR thymocyte proliferation assay[1]. Intracellular and extracellular IL-1 activities were expressed as dpm of [^{3}H]TdR incorporated by thymocytes at 1:8 dilution.

Phagocytic activity determination Phagocytosis was determined employing the method of neutral red[7] with minor modifications. Murine exudate cells primed 4 d previously were added at 2×10^5/well. Macrophages were separated by adherence on the plates. Various concentrations of Tri were added to the adherent macrophages in a volume of 0.1 RPMI-1640 supplemented with 10% FCS. After 24 h of incubation, the media was discarded and 0.1 ml of 0.1% neutral red in PBS was added to each well. The cells were incubated at 37 C for 30 min. The neutral red was removed and the cells were washed twice by PBS. Extraction solution 0.1 ml (EtOH : HAc = 1 : 1) was added to each well to extract the dye taken by macrophages and absorbance of the color was read on a DG 3022 microplate spectrophotometer (Xi-an, China) at 550 nm. The phagocytic activtiy was expressed as the amount of neutral red phagocytized by macrophages.

Statistical analysis All data were expressed as $\bar{x} \pm s$ and analyzed by t test.

RESULTS

Mitogen-induced mouse splenocyte proliferation The levels of proliferation were determined by measuring the radioactivity of [^{3}H]TdR. Tri 0.01—10 $\mu mol \cdot L^{-1}$ inhibited the splenocyte proliferation induced by Con A 5 $\mu g \cdot ml^{-1}$ or LPS 10 $\mu g \cdot ml^{-1}$ concentration-dependently (Tab 1).

Tab 1. Effect of triazolodiazepine (Tri) on mouse splenocyte proliferation induced by concanavalin A (Con A 5 $\mu g \cdot ml^{-1}$) or lipopolysaccharides (LPS 10 $\mu g \cdot ml^{-1}$). $n = 6$ samples. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ vs 0.

Tri/$\mu mol \cdot L^{-1}$	Radioactivity/dpm	
	Con A	LPS
0	106 037±9 443	7 654±645
0.001	91 407±7 142^b	7 074±627^a
0.01	88 205±14 021^b	6 212±566^c
0.1	68 554±5 605^c	4 617±543^c
1	63 458±4 241^c	4 221±606^c
10	28 378±3 851^c	1 504±214^c

Production of CSF by mouse splenocytes CSF activity was determined by BMC proliferation assay. Con A 5 $\mu g \cdot ml^{-1}$-induced CSF activity at different dilutions was obviously reduced by Tri at 10 $\mu mol \cdot L^{-1}$ but not at lower concentrations (Fig 1).

IL-1 release and synthesis When added to LPS-stimulated adherent peritoneal macrophages, Tri markedly inhibited the

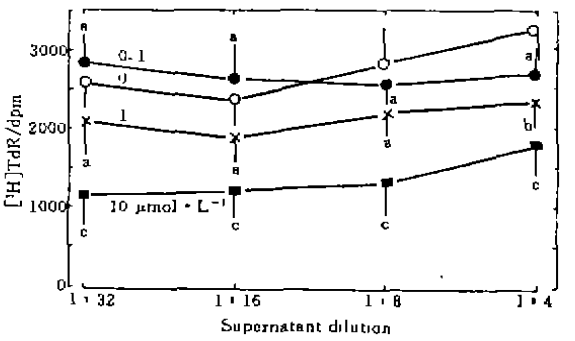

Fig 1. Effect of Tri on production of colony-stimulating factor (CSF) from mouse splenocytes induced by Con A 5 μg·ml⁻¹. $n=6$, $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* 0.

release of IL-1 in a concentration-dependent manner. Similar decrease was obtained in the cell-associated IL-1 activity (Tab 2). The intracellular production of IL-1 was less than extracellular one.

Tab 2. Inhibitory effect of Tri on LPS-induced production of IL-1 from mouse peritoneal macrophages, expressed as [³H]TdR incorporation by mouse thymocytes at 1:8 dilution. $n=3$ samples. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* 0.

Tri/μmol·L⁻¹	Radioactivity/dpm	
	Intracellular	Extracellular
0	13 852±1 758	20 954±4 231
0.001	11 645±1 696[a]	16 885±2 003[a]
0.01	10 692±2 546[b]	13 473±523[b]
0.1	10 421±878[b]	11 596±2 664[b]
1	4 823±1 390[c]	8 832±921[c]
10	5 824±1 313[c]	5 770±265[c]

Phagocytosis Phagocytic activity of activated macrophages was presented as amount of neutral red taken by macrophages. Tri (> 0.1 μmol·L⁻¹) exerted an inhibitory effect on phagocytosis of mouse peritoneal macrophages (Tab 3).

Tab 3. Effect of Tri on phagocytic activity of activated mouse peritoneal macrophages, expressed as the amount of neutral red phagocytized by macrophages. $n=6$ samples. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* 0.

Tri/μmol·L⁻¹	Neutral red/μg
0	11.26±1.78
0.001	11.06±1.58[a]
0.01	10.46±2.57[a]
0.1	9.04±0.99[b]
1	7.83±1.57[c]
10	7.45±0.77[c]

DISCUSSION

Tri markedly inhibited most of PAF response and elicited strong anti-inflammatory effects. In this paper Tri inhibited Con A and LPS-induced murine spleen cell proliferation. Con A and LPS induced the proliferation of T and B lymphocytes, respectively. Ward SG *et al* reported Tri had no effects on mitogen or interleukin-2-induced human T lymphocyte proliferation[8]. Different cell resources may be explained as the cause of this contradiction. CSF not only stimulates the proliferation and differentiation of both myelomonocytic and erythroid stem cells, but also induces a significant leukotrienes synthesis and enhances arachidonic acid release, amplifying the inflammatory response[9]. IL-1 is also an important mediator in inflammatory reactions. In this study Tri reduced Con A-induced CSF release from splenocytes and LPS-stimulated IL-1 production from peritoneal macrophages. Phagocytic activity is one of the important characteristics of macrophages[7]. Phagocytosis takes part in the beginning of various immunological response. Tri inhibited the phagocytic activity of activated macrophages. This article does not determine the precise mechanism of this action. The inhibition of immunological process including T, B lymphocyte proliferation, macrophage phagocytosis and cytokine production may partially explain

its wide and strong anti-inflammatory effects.

PAF-acether increased release, synthesis and total production of IL-1 by rat macrophages. this effect of PAF was inhibited in the presence of the specific antagonist, BN 52021. Challenged by LPS, macrophages produced both IL-1 and PAF[5,10]. These results suggest that PAF plays a key role in the production of IL-1. Our previous data showed that Tri decreased the production of tumor necrosis factor (TNF) by macrophages[6]. PAF is a major inflammatory mediator which may interact with other mediators like IL-1, CSF and TNF, thereby resulting in an autocatalytic augmentation of the inflammatory response[2]. Tri, being able to inhibit the production of these cytokines, may be of great importance in anti-inflammatory therapy.

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Triazolodiazepine 在体外对小鼠脾细胞和腹腔巨噬细胞的抑制作用

R965.2

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摘要 Triazolodiazepine (Tri)体外对 Con A 和 LPS 诱导的小鼠脾细胞增殖以及巯基乙醇硫酸盐激活的腹腔巨噬细胞吞噬功能呈浓度依赖性抑制作用. Tri 还明显抑制 Con A 诱导的脾细胞释放集落刺激因子和巨噬细胞释放白细胞介素-1，结果提示，Tri 抑制小鼠脾细胞和巨噬细胞功能可能与其广泛而强大的抗炎作用有关.

Tri

关键词 triazolodiazepine；白细胞介素-1；集落刺激因子；巨噬细胞；吞噬作用；脾脏