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Effects of triazolodiazepine on the production of interleukin-6 from murine spleen cells and rabbit synovial cells *in vitro*

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INTERLEUKIN-6 (IL-6) is a multifunctional cytokine that regulates the immune response, acute phase anaphylactic reaction, and haematopoiesis. Lipopolysaccharide (6–24 μg/ml) significantly induced IL-6 release from murine spleen cells. In cultured rabbit synovial cells interleukin-1 (IL-1, 1–10 U/ml) induced IL-6 production in a concentration-dependent manner. Triazolodiazepine (Tri) is a hetrazepine platelet-activating factor antagonist. In this study we found that Tri (0.1–10 μmol/l) exerted strong inhibitory effects on LPS stimulated IL-6 production in murine spleen cells. Kinetic studies showed that the inhibition of IL-6 release was time-independent. In rabbit synovial cells Tri also reduced IL-6 release induced by IL-1 and tumour necrosis factor. Inhibition of cytokine production by Tri may partially explain its wide and strong anti-inflammatory effects.

Key words: IL-6, Spleen cells, Synovial cells, Triazolodiazepine

Introduction

Interleukin-6 (IL-6) is a multifunctional cytokine that regulates the immune response, acute phase anaphylactic reaction, and haematopoiesis.[1] It is known that IL-6 is produced by various cells.[2] Considering that IL-6 production may play a critical role in a number of disease, chronic inflammation, and lymphoid malignancies, relatively little is known about the effects of anti-inflammatory drugs on cytokine synthesis. It might therefore be worthwhile investigating the relationship between its inhibition and the mechanism of action of anti-inflammatory drugs. Triazolodiazepine (Tri) is a hetrazepine platelet-activating factor (PAF) antagonist. It exerts strong anti-inflammatory effects on many *in vivo* and *in vitro* models.[3] In the present study, the effects of Tri on the production of IL-6 from mouse splenocytes and cultured rabbit synovial cells were examined.

Materials and Methods

Animals and chemicals: ICR mice weighing 16–24 g and New Zealand rabbits weighing 1.5–2.5 kg were purchased from the Zoological Experiment Center of the Second Military Medical University. RPMI-1640, MEM media and lipopolysaccharide (LPS, *Escherichia coli*, 055:B5) were purchased from Sigma Chemical Co., USA. Foetal calf serum (FCS) was supplied by the Department of Pathology, Second Military Medical University. Triazolodiazepine (Tri, WEB 2086) was a kind gift from Boehringer Ingelheim Co., Germany. [^{3}H]-TdR (814 TBq/mol) was obtained from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. Recombinant human tumour necrosis factor (TNF) was generously provided by Dr Y. Sohmura, Dainippon Pharmaceutical Co., Japan.

Spleen cell preparation and IL-6 induction: Splenocytes were prepared from spleens removed under sterile conditions from sacrificed ICR mice.[4] The cells were suspended in RPMI-1640 containing penicillin 100 U/ml, streptomycin 100 μg/ml, 2-mercaptoethanol 50 mmol/l and 10% FCS. The cells were cultured in sterile 24-well tissue culture plates (Corning, USA) at a density of 5×10^6 cells/ml/well with LPS alone or in combination with different concentrations of Tri. After 48 h incubation at 37°C in a 5% CO_2, humidified atmosphere, the supernatants were collected by centrifugation and stored −20°C until testing for IL-6 activity.

IL-6 production from rabbit synovial cells in culture: Rabbit synovial cells were cultured according to Ju.[5] Briefly, synovial membrane tissue was obtained aseptically from the knees of normal adult New Zealand rabbits. The membrane was cut into pieces and stuck to a culture flask with the inner side towards the flask wall. MEM medium supplemented with 10% FCS, penicillin (100 U/mol) and streptomy-

cin (100 μg/ml) were added to the flask and cultivation was carried out at 37°C. When the cells had grown to confluence, they were trypsinized and seeded into a 24-well plate. Twenty-four hours later, the compounds to be tested were added to the cell monolayer. After 24 h incubation the supernatants were collected by centrifugation and stored at −20°C for IL-6 assay.

IL-6 bioassay: To measure IL-6 activity in the supernatants, the murine IL-6 dependent hybridoma cell line 7TD1 was used.[6] Briefly, cells were cultured on culture flasks in RPMI-1640 with 10% FCS and recombinant human IL-6 (gifts from Dr Steven Gillis, Immunex Co., USA). 7TD1 cells were seeded into 96-well flat-bottomed microtitre plates (Nunclon, Denmark) at a density of 1000 cells/well. Supernatants to be tested were added to the cells and incubated for 3 days in a humidified atmosphere. Cells were pulsed with [^{3}H]-TdR 18500 Bq for the last 12 h and were collected using a cell harvester. The incorporation of [^{3}H]-TdR was measured using a FJ-2107 scintillation counter (Xi-an, China).

Results

IL-6 production induced by LPS: The levels of IL-6 production were determined by measuring the radioactivity of [^{3}H]-TdR incorporation in 7TD1 cells. LPS at 6–12 μg/ml markedly induced IL-6 production from murine spleen cells (Fig. 1).

Inhibition of Tri on IL-6 production from splenocytes: Splenocytes were incubated with LPS and Tri for 48 h. The release of IL-6 induced by LPS (12 μg/ml) was shown to be significantly reduced by Tri at 0.1–10 μmol/l in a concentration-dependent manner as indicated in Fig. 2.

Time course of IL-6 production: Tri co-cultured with LPS stimulated splenocytes for 24, 36, 48, 60 and 72 h. The data in Fig. 3 shows that at all times tested,

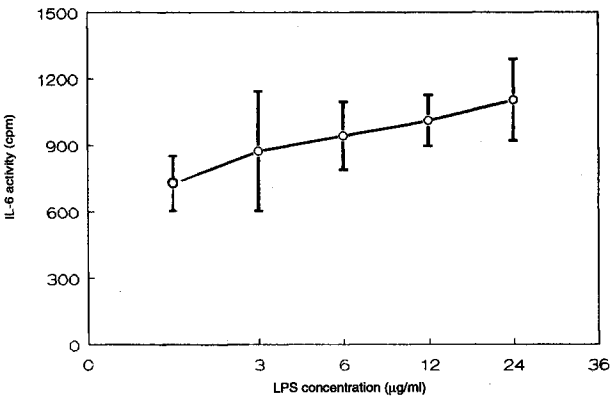

FIG. 1. Effect of lipopolysaccaride (LPS) on the production of interleukin-6 (IL-6) in mouse splenocytes. x ± S.D., $n = 6$, * $p < 0.05$, ** $p < 0.01$ *vs* control.

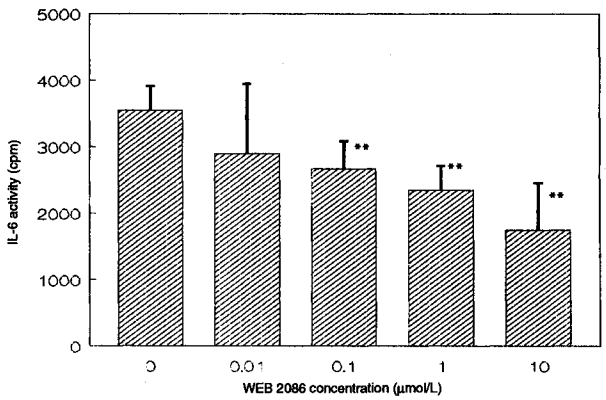

FIG. 2. Inhibitory effects of triazolodiazepine (Tri) on LPS (12 μg/ml) stimulated IL-6 production in murine splenocytes. x ± S.D., $n = 6$, * $p < 0.01$ *vs* control.

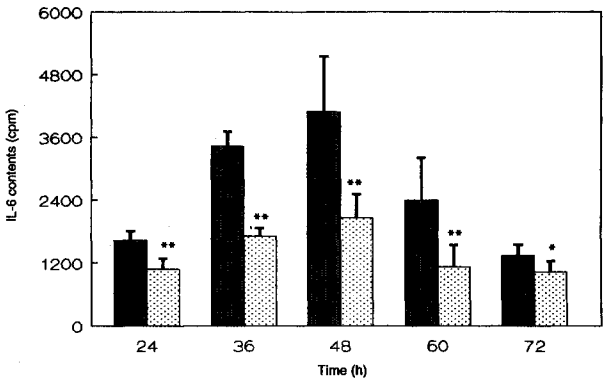

FIG. 3. Time-independent inhibition of Tri (10 μmol/l) on the production of IL-6 in mouse splenocytes. x ± S.D., $n = 6$, * $p < 0.05$, ** $p < 0.01$ *vs* LPS group. ■, LPS alone; ▨, LPS+WEB 2086.

Tri (10 μmol) elicited strong inhibitory effects on IL-6 production in murine spleen cells, suggesting that Tri inhibited IL-6 production in a time-independent manner.

IL-6 production from synovial cells: Rabbit synovial cells were stimulated with IL-1 and TNF for 24 h. The results demonstrated that IL-1 at 10 and 100 U/ml, TNF at 1 and 10 U/ml both significantly stimulated IL-6 release from synovial cells. The inducing effects seemed to be concentration-dependent (Table 1).

Table 1. Effects of tumour necrosis factor (TNF) and interleukin-1 (IL-1) on IL-6 production from cultured rabbit synovial cells. x ± S.D., $n = 6$, * $p < 0.05$, ** $p < 0.01$ *vs* control

Drugs U/ml	IL-6 activity (cpm)
Control	2081 ± 328
IL-1	
1	2217 ± 256
10	2533 ± 374*
100	3450 ± 476**
TNF	
0.1	2398 ± 522
1	2870 ± 513**
10	4253 ± 631**

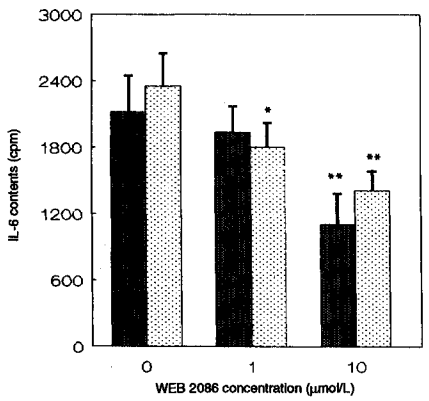

FIG. 4. Inhibitory effects of Tri on the production of IL-6 induced by IL-1 (100 U/ml) and TNF (10 U/ml) from rabbit synovial cells. x ± S.D., $n = 6$, $^*p < 0.05$, $^{**}p < 0.01$ *vs* control. ■, IL-1 (100 U/ml); ▨, TNF (10 U/ml).

Reduction of IL-6 production from synovial cells by Tri: IL-6 release challenged by IL-1 (100 U/ml) was markedly reduced by Tri (10 μmol/l). Tri also inhibited TNF (10 U/ml) induced IL-6 production at 1 and 10 μmol/l (Fig. 4).

Discussion

IL-6 is known to have multiple actions on the growth, differentiation and function of lymphoid and non-lymphoid cells. It has recently been suggested that this cytokine plays a role in the pathogenesis of autoimmune disorders, in which excessive production of IL-6 may lead to abnormal B-cell differentiation and antibody production.[7] This study demonstrates that LPS significantly induced IL-6 production from murine spleen cells *in vitro*. Since splenocyte culture is a mixture of T-cells, B-cells and macrophages, the data here do not provide direct insight into the cellular source of IL-6. It is reported that macrophages, fibroblasts and endothelial cells are the main IL-6 producing cells,[8] but we also found that IL-1 and TNF stimulated IL-6 production in cultured rabbit synovial cells. IL-6 release from synovial cells in arthritic DBA/ij mice was reported by Sugita *et al.*[9] Elevation of IL-6 levels in sera and paws of autoimmune arthritic animals was also demonstrated.[10] In previous work it was found that IL-6 stimulated proliferation of synovial cells.[5] These data suggest that abnormal IL-6 production may be an important pathological parameter and a contributory factor in the proliferative lesions of arthritic disease.

Tri is a specific PAF antagonist. We found that Tri reduced the production of TNF from murine peritoneal macrophages, IL-1 and colony-stimulating factor from mouse splenocytes.[4,11] In this study we found that Tri exerted strong inhibitory effects on IL-6 production from LPS stimulated murine spleen cells and cultured rabbit synovial cells. We speculate that PAF may play an essential role in the production of these cytokines. PAF is a major inflammatory mediator which may interact with other cytokines such as IL-1, IL-6, CSF and TNF, thereby resulting in an autocatalytic augmentation of the inflammatory response.[3,12–14] Tri, being able to exhibit strong inhibitory effects on the production of these mediators, may be of great use in anti-inflammatory therapy.

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