

Inhibitory Effect of T-614 on Tumor Necrosis Factor- α Induced Cytokine Production and Nuclear Factor- κ B Activation in Cultured Human Synovial Cells

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ABSTRACT. *Objective.* To investigate the mechanism of the immunosuppressive effect of T-614 [*N*-(3-formylamino-4-oxo-6-phenoxy-4*H*-chromen-7-yl)methanesulfonamide], a new antirheumatic drug whose clinical efficacy has been determined for the treatment of patients with rheumatoid arthritis (RA). *Methods.* RA synovial fibroblast-like cells were cultured with tumor necrosis factor- α (TNF- α , 10 ng/ml) in the presence or absence of T-614. After incubation, cytokine production was measured by ELISA. Expression of interleukin 6 (IL-6) and IL-8 mRNA was examined by real-time quantitative reverse transcriptase-polymerase chain reaction analysis and TNF- α induced nuclear factor- κ B (NF- κ B) activation was observed using immunostaining with an antibody against NF- κ B p65. *Results.* T-614 suppressed TNF- α induced production of IL-6, IL-8, and monocyte chemoattractant protein 1, and also reduced the accumulation of IL-6 and IL-8 mRNA in a concentration dependent manner. T-614 interfered with the TNF- α induced translocation of NF- κ B to the nucleus from the cytoplasm. *Conclusion.* Inhibition of NF- κ B activation and transcription of proinflammatory cytokines by T-614 contributes to its clinical antirheumatic effect. (J Rheumatol 2001;28:2591–6)

Key Indexing Terms:

RHEUMATOID ARTHRITIS

TUMOR NECROSIS FACTOR- α

SYNOVIAL CELLS

NUCLEAR FACTOR- κ B

CHEMOKINE

T-614

Rheumatoid arthritis (RA) is an autoimmune inflammatory disease that destroys the synovial joints¹. Synovial tissue of patients with RA is characterized by the infiltration of activated lymphocytes and macrophages, and the proliferation of synovial cells². Various cytokines produced by the infiltrating cells and synovial cells are the underlying cause of many processes in RA¹. Among these, tumor necrosis factor- α (TNF- α) is essential for the induction of other proinflammatory cytokines such as interleukin 6 (IL-6), IL-8, and monocyte chemoattractant protein 1 (MCP-1) from synovial cells^{1,3,4}. Further, the participation of TNF- α in the symptoms of RA has been demonstrated by marked clinical improvement in anti-TNF- α therapy with monoclonal antibody directed to TNF- α ⁵.

The action of TNF- α is mainly mediated by the activation of nuclear factor- κ B (NF- κ B)⁶. NF- κ B is complexed with an

inhibitor I κ B in the cytoplasm of resting cells. When these are stimulated, NF- κ B is released from I κ B, which has been phosphorylated and degraded, translocates to the nucleus, binds to NF- κ B sites in target genes such as IL-6, IL-8, and MCP-1, and activates their transcription^{7–11}.

T-614 [*N*-(3-formylamino-4-oxo-6-phenoxy-4*H*-chromen-7-yl)methanesulfonamide] is a new antirheumatic drug that currently is undergoing phase III clinical trials in Japan. It has been reported that T-614 significantly inhibited the release of IL-1 and IL-6 from peripheral monocytes *in vitro*¹², reduced the cartilage and bone damage in type II collagen induced arthritis in DBA/1J mice, and suppressed the development of spontaneous arthritis in MRL/l mice¹³. Further, it has been shown that oral administration of T-614 to rats leads to clinical improvement of experimental autoimmune encephalomyelitis, and its inhibitory action on TNF production by the pathogenic T cells is related to this improvement¹⁴.

We investigated the clinical antirheumatic action of T-614, examining its effect on synovial cell responses to TNF- α using cultured synovial cells obtained from patients with RA.

MATERIALS AND METHODS

*Establishment of synovial cells*¹⁵. Synovial tissues were collected from patients with RA at the time of total joint replacement. Synovial tissues were minced into small pieces and treated with 1 mg/ml of collagenase for 2 h at 37°C in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan), then were washed twice in the medium. Then the cells were suspended in RPMI 1640 medium containing 10% fetal bovine serum (Biowhittaker, Walkersville, MD, USA), 100 U/ml penicillin, and 100 μ g/ml streptomycin

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(Life Technologies, Rockville, MD, USA) (FBS-RPMI 1640). These cell suspensions were dispensed into 80 cm² culture flasks (Nunc, Roskilde, Denmark) and cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air. When the cell cultures reached confluence, synovial cells were trypsinized and passaged to other flasks. The cells, synovial fibroblast-like cells, between the 4th and 10th passage were used for the experiments. All patients with RA met the American College of Rheumatology 1987 revised criteria¹⁶.

Measurement of cytokine production. The synovial cells from 3 individuals with RA were seeded in 96 well flat bottom microtiter plates (Nunc) at density 5 × 10³ cells/well and cultured 48 h at 37°C. Triplicate cultures were then prepared by adding FBS-RPMI 1640 containing T-614 at concentrations of 0, 3, or 30 µg/ml. After 4 h incubation, recombinant human TNF-α (Genzyme, Cambridge, MA, USA) at concentration 10 ng/ml was added and the cells were incubated in a total volume of 100 µl for another 72 h period. Next, the culture supernatants were recovered and stored at -80°C until the cytokine measurements were performed. Cell viability was determined using a modified MTT assay (WST-1 assay; Dojindo, Kumamoto, Japan)¹⁵. Concentrations of IL-6, IL-8, and MCP-1 were measured using the respective commercial ELISA kits (BioSource International, Camarillo, CA, USA).

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR). Synovial cells (2 × 10⁶ cells/dish) in a 10 cm culture dish (Costar, Corning, NY, USA) were cultured in FBS-RPMI 1640 in the presence or absence of T-614 (0.3, 3, or 30 µg/ml). After 1 h, TNF-α (10 ng/ml) was added and the culture continued for another 24 h. The cells were then harvested with a plastic scraper and total cellular RNA was isolated from the collected cells using Isogen guanidium thiocyanate¹⁷ (Nippon Gene, Tokyo, Japan). Precipitated RNA was reverse transcribed by the SuperScript Preamplification System (Life Technologies) according to the manufacturer's instructions. PCR amplification was performed for 18 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. PCR primer sets used for amplification were as follows; 5'-ATGACTTCCAAGCTGGCCGTG-3' and 5'-TTATGAATTCTCAGCCCTCTTCAAAAACCTTCTC-3' for IL-8¹⁸ (human IL-8 primer set; Maxim Biotech, San Francisco, CA, USA), which amplified a 300 bp fragment; and 5'-CCACCCATGGCAAATTCATGGCA-3' and 5'-TCTAGACGGCAGGTCAGGTCCACC-3' for GAPDH¹⁹ (human GAPDH primer set for RT-PCR; Stratagene, La Jolla, CA, USA), which amplified a 600 bp fragment. The mixture of each PCR product was electrophoresed in 2% agarose gel (Nippon Gene) and stained with ethidium bromide.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR analysis was performed starting with 0.5 µg of total RNA as described above using Taqman[®] reverse transcription reagents and predeveloped Taqman[®] assay reagents (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol in an ABI Prism 7700 sequence detection instrument (PE Applied Biosystems). The relative amount of IL-6 and IL-8 transcripts in each sample was determined by normalizing for GAPDH mRNA expression levels, as described in the ABI Prism 7700 sequence detection system user's manual.

Immunohistochemistry for NF-κB. Immunoperoxidase staining was performed at room temperature with the Vectastain ABC kit (Vector, Burlingame, CA, USA) according to the manufacturer's suggested protocol²⁰. Adherent synovial fibroblast-like cells were plated in LabTek chamber slides (Nunc) at density 3 × 10⁴ cells/chamber in FBS-RPMI 1640 at 37°C for 2 days. Next, the cells were incubated with or without 30 µg/ml of T-614 for 1 h and with 10 ng/ml of TNF-α for another 1.5 h period. After incubation, supernatants were discarded and the slides were fixed with acetone for 30 s and immersed in 0.3% peroxidase in methanol for 45 min to deplete endogenous peroxidase. Fifty micrograms per milliliter of primary antibodies against NF-κB p65²¹ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or normal goat IgG were applied to the slides for 30 min at room temperature. After washing in PBS, biotinylated rabbit anti-goat IgG (Vector) was applied to the slides, followed by incubation with avidin DH-biotinylated peroxidase (Vector) for 45 min. Finally, the slides were immersed in a peroxidase substrate solution containing 0.05% of 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.04% of nickel chloride, and 0.01% of hydrogen for 5 min. Counterstaining was per-

formed with Mayer's hematoxylin (Wako Pure Chemical). Positive staining was indicated as a brownish black.

Statistical analysis. Data are presented as mean ± SEM. Statistical analysis was performed using Dunnett's test, comparing all T-614 concentrations with the control. A probability < 0.05 was defined as significant.

RESULTS

Effect on TNF-α induced production of IL-6, IL-8, and MCP-1. We examined the effect of T-614 on TNF-α induced cytokine production by synovial cells. The cells were stimulated with 10 ng/ml of TNF-α in the presence or absence of T-614 for 72 h and culture supernatants were recovered for measurement of cytokine production. As shown in Figures 1, 2, and 3, synovial cells produced a large amount of IL-6, IL-8, and MCP-1 in response to TNF-α, although small amounts of IL-6 and MCP-1 were detected in the culture supernatant of unstimulated cells. Treatment of cells with T-614 at concentrations of 3 or 30 µg/ml significantly suppressed TNF-α induced production of these 3 cytokines. MCP-1 production by unstimulated cells was decreased by T-614 at 30 µg/ml. In addition, T-614 did not affect cell viability estimated by modified MTT assay (data not shown).

Effect on TNF-α induced IL-8 and IL-6 mRNA expression. To examine whether T-614 suppresses gene transcription, we carried out a conventional and/or real-time quantitative RT-PCR analysis for IL-8 and IL-6 mRNA. Synovial cells were incubated with TNF-α 10 ng/ml and T-614 for 24 h, and then the total RNA was extracted. The appropriate number of PCR

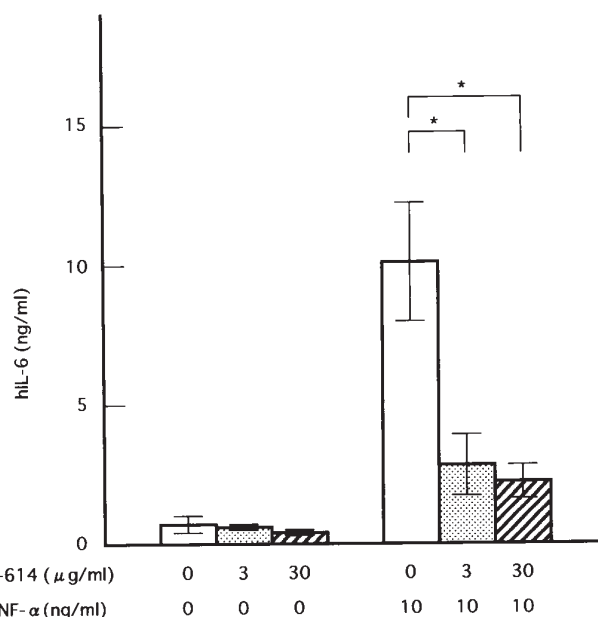


Figure 1. Effect of T-614 on TNF-α stimulated production of IL-6. Synovial fibroblast-like cells were incubated with various concentrations (0, 3, 30 µg/ml) of T-614 and stimulated by TNF-α (10 ng/ml) for 72 h. Supernatants were recovered and production of IL-6 was measured by ELISA. TNF-α stimulated production of IL-6 was suppressed by T-614. *p < 0.05. Data are shown as mean ± SEM of 3 independent experiments using different cell lines.

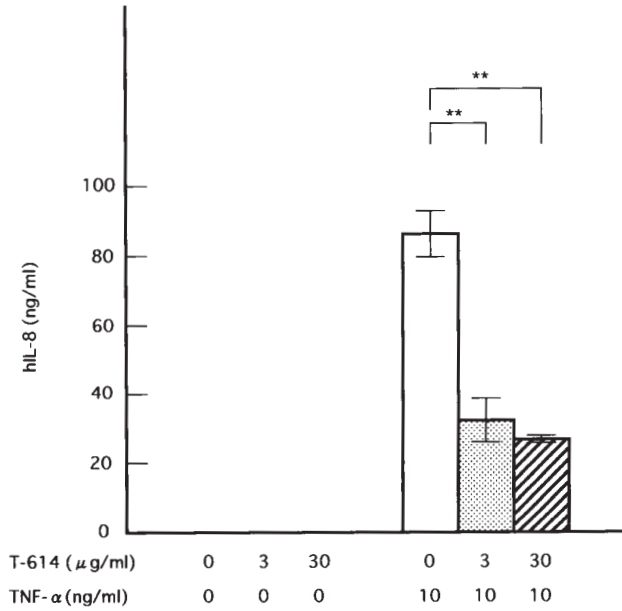


Figure 2. Effect of T-614 on TNF- α stimulated production of IL-8. Synovial fibroblast-like cells were incubated with various concentrations (0, 3, 30 μ g/ml) of T-614 and stimulated by TNF- α (10 ng/ml) for 72 h. Supernatants were recovered and production of IL-8 was measured by ELISA. TNF- α stimulated production of IL-8 was significantly suppressed by T-614. ** $p < 0.01$. Data are shown as mean \pm SEM of 3 independent experiments using different cell lines.

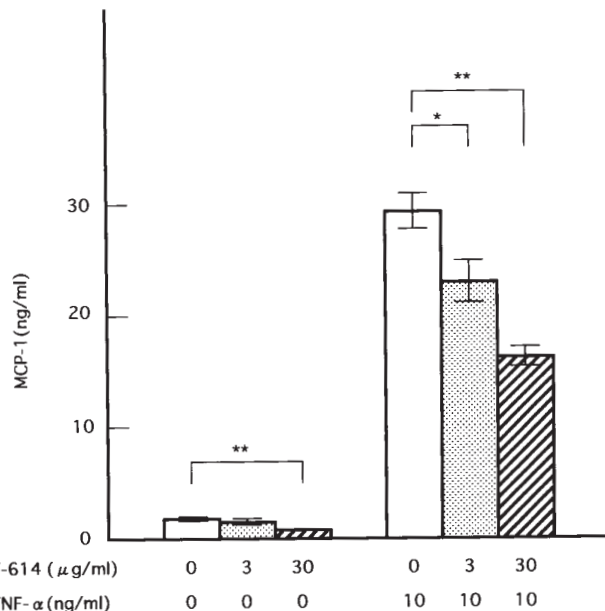


Figure 3. Effect of T-614 on TNF- α stimulated production of MCP-1. Synovial fibroblast-like cells were incubated with various concentrations (0, 3, 30 μ g/ml) of T-614 and stimulated by TNF- α (10 ng/ml) for 72 h. Supernatants were recovered and production of MCP-1 was measured by ELISA. MCP-1 production stimulated by TNF- α was suppressed by T-614 in a concentration dependent manner, and when there was no TNF- α stimulation, 30 μ g/ml of T-614 suppressed MCP-1 production. * $p < 0.05$, ** $p < 0.01$. Data are shown as mean \pm SEM of 3 independent experiments using different cell lines.

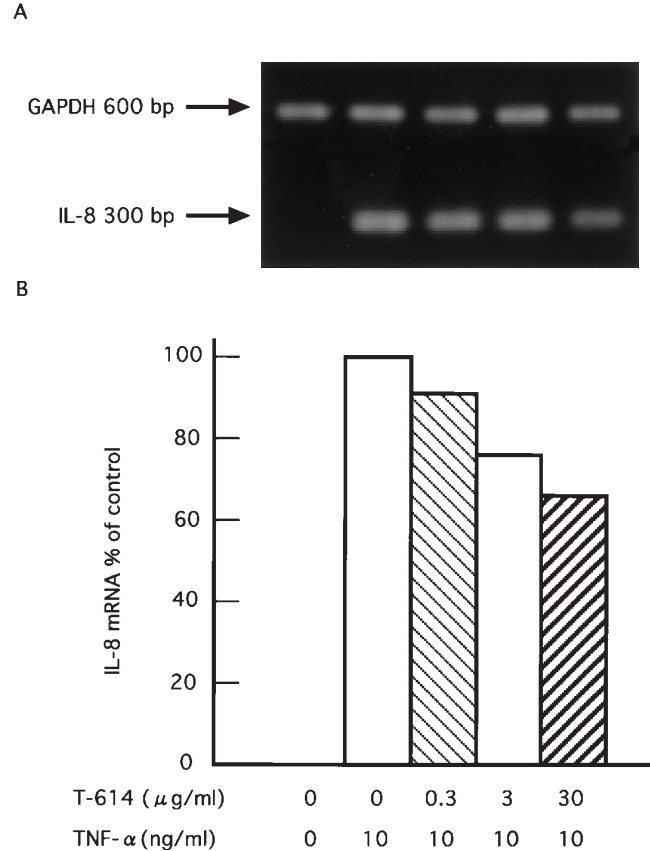


Figure 4. Effect of T-614 on TNF- α stimulated expression of IL-8 mRNA. After incubation of synovial fibroblast-like cells (2×10^6 cells) with various concentrations (0, 0.3, 3, 30 μ g/ml) of T-614 and stimulation by TNF- α (10 ng/ml) for 24 h, conventional RT-PCR was performed for IL-8 and GAPDH mRNA (A). Real-time RT-PCR analyses for same samples were performed and relative amount of IL-8 was determined by normalizing for GAPDH mRNA expression levels (B). T-614 reduced TNF- α stimulated accumulation of IL-8 mRNA in a concentration dependent manner.

cycles was estimated with regard to IL-8 and GAPDH cDNA, which were synthesized by reverse transcription. As shown in Figure 4A, target bands for IL-8 from T-614 treated cells were decreased compared with controls. Furthermore, real-time quantitative RT-PCR analysis for the same samples revealed that T-614 reduced TNF- α induced accumulation of IL-8 mRNA in a concentration dependent manner (Figure 4B) and strongly suppressed IL-6 mRNA expression (Figure 5).

Effect of T-614 on the activation of NF- κ B by TNF- α . Since T-614 inhibited transcription, we investigated whether T-614 interferes with NF- κ B activation. Synovial cells were stimulated with 10 ng/ml TNF- α in the presence or absence of 30 μ g/ml of T-614 for 1.5 h, and the localization of p65 protein was detected by immunostaining using the antibody against NF- κ B p65. Synovial cells stimulated with TNF- α revealed NF- κ B immunostaining in both cytoplasm and nucleus (Figure 6A). In contrast, the cells incubated with 30 μ g/ml of T-614 revealed immunostaining only in the cytoplasm, but not in the nucleus (Figure 6B).

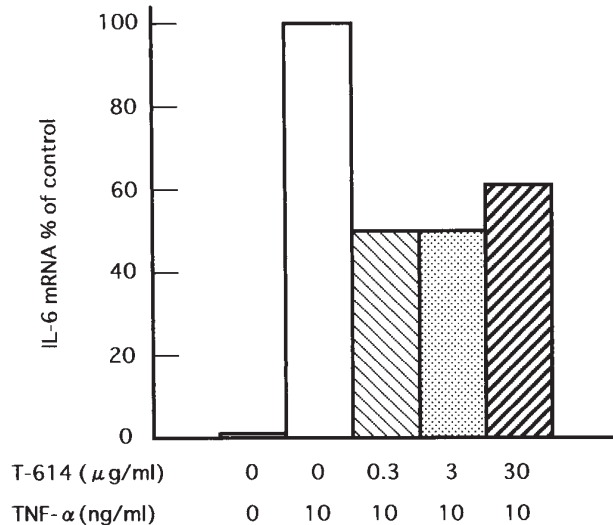


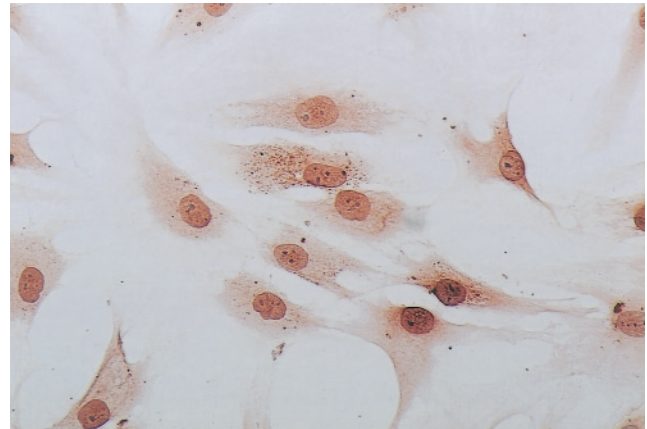
Figure 5. Effect of T-614 on TNF- α stimulated expression of IL-6 mRNA. After incubation of synovial fibroblast-like cells (2×10^6 cells) with various concentrations (0, 0.3, 3, 30 $\mu\text{g/ml}$) of T-614 and stimulation by TNF- α (10 ng/ml) for 24 h, real-time quantitative RT-PCR analysis was performed and the relative amount of IL-6 was determined by normalizing for GAPDH mRNA expression levels. T-614 reduced TNF- α stimulated accumulation of IL-6 mRNA.

DISCUSSION

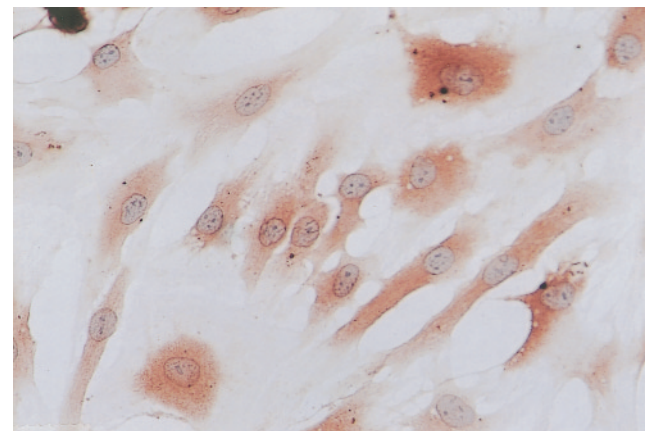
We observed that T-614 suppressed TNF- α induced production of IL-6, IL-8, and MCP-1, and reduced the accumulation of IL-6 and IL-8 mRNA in a concentration dependent manner. We also found that T-614 interfered with the TNF- α induced translocation of NF- κB to the nucleus from the cytoplasm. These data suggest that the clinical antirheumatic effect of T-614 is accomplished by inhibition of NF- κB activation and transcription of proinflammatory cytokines.

T-614 is a new antirheumatic drug, which possesses antiarthritic, antiinflammatory, analgesic, and antipyretic profiles²²⁻²⁴, and has been shown to improve clinical and laboratory indices in patients with RA²⁵. A recent study reported that in cultured synovial cells obtained from patients with RA, T-614 inhibited the upregulated expression of some costimulatory molecules (CD54 and CD106) and cytokine production (IL-6, IL-8, granulocyte-colony stimulating factor) at a concentration of 2 $\mu\text{g/ml}$ when the cells were exposed to interferon- γ , IL-1 β , or 12-O-tetradecanoyl phorbol 13-acetate²⁶. However, the effect of T-614 on synovial cell responses to TNF- α , which is thought to be a key cytokine in pathogenesis of RA, was unknown. We therefore examined its effect on TNF- α induced cytokine production by synovial cells and the activation process of NF- κB that has been reported to play an important role in the cellular signal pathway from the receptor on the cell surface to the nucleus⁷.

As a result, T-614 suppressed the production of IL-6, IL-8, and MCP-1 by synovial fibroblast-like cells stimulated with TNF- α . Our findings also indicated that TNF- α induced IL-6 and IL-8 gene expressions were suppressed by T-614 in a con-



A



B

Figure 6. Effect of T-614 on the activation of NF- κB by TNF- α . The TNF- α induced NF- κB p65 protein was detected with immunostaining using antibody against NF- κB p65. This examination was performed on 2 different cell lines. The representative result is shown. A. Synovial cells stimulated by TNF- α (10 ng/ml) revealed NF- κB immunostaining in both the cytoplasm and nucleus. B. Synovial cells stimulated with TNF- α (10 ng/ml) and incubated with T-614 (30 $\mu\text{g/ml}$) revealed NF- κB immunostaining in cytoplasm only, not in the nucleus.

centration dependent manner. Single binding sites for NF- κB are present in the promoter of the IL-6^{11,27}, IL-8^{10,12,28,29}, and MCP-1^{13,30,31} genes, hence the induction of these cytokines is controlled by NF- κB . It has been reported that NF- κB is expressed in the rheumatoid synovium³² and stimulation of synovial cells with TNF- α activates NF- κB and subsequent transcription of several genes³³. In this study, immunohistochemistry of synovial cells showed that T-614 interferes with the TNF- α induced translocation of NF- κB to the nucleus from the cytoplasm.

The concentration (30 $\mu\text{g/ml}$) of T-614 inhibiting the cytokine production and NF- κB activation somewhat exceeded the pharmacological levels when administered to humans. T-614 is usually administered at a dose of 50–100 mg/day to

adults, and plasma concentrations of T-614 are estimated to reach approximately 3 µg/ml (data on file, Toyama Chemical Co., Ltd.). The concentration (10 ng/ml) of TNF-α used as a stimulus in this study would also be higher than physiological levels, which have been reported to be about 300 pg/ml in synovial fluid of patients with RA³⁴. If TNF-α was added at a lower concentration, the effect of T-614 might appear at a lower dose. Therefore, the sufficient efficacy of T-614 on cytokine production would be expected in clinical study.

Nonsteroidal antiinflammatory drugs are generally believed to exert their antiinflammatory effect through the inhibition of cyclooxygenase (COX) activity³⁵. Recent studies have indicated that the COX-2 gene has transcriptional regulatory sequences of 2 NF-κB sites^{36,37}. It was reported that T-614 reduced prostaglandin E₂ content in the inflammatory exudate of rats *in vivo*²³. Its inhibitory effect on prostanoid production is mediated by the synergy of not only the mild inhibition of COX-2 activity, but also the inhibition of COX-2 induction³⁸, the mechanism of which has been unclear. Our results suggest that the inhibition of COX-2 induction by T-614 might be through its inhibitory action on NF-κB. Recently, we reported that auranofin (2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranosato-S) inhibits IL-1β induced prostaglandin E₂ synthesis and COX-2 expression via the suppression of NF-κB activation of synoviocytes²¹.

We suggest a possible mechanism for the antirheumatic action of T-614. Our findings show that the clinical effect of T-614 on RA is via the inhibition of translocation of NF-κB from the cytoplasm to the nucleus and by blocking transcription of proinflammatory cytokines such as IL-6 and IL-8.

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