Mod Rheumatol (2001) 11:28–33

ORIGINAL ARTICLE

Eiji Sugiyama · Masahito Ikemoto · Hirofumi Taki
Muneharu Maruyama · Naohiro Yamashita
Masashi Kobayashi

Mizoribine, an inhibitor of inosine monophosphate dehydrogenase, inhibits interleukin-6 production by freshly prepared rheumatoid synovial cells

Received: April 4, 2000 / Accepted: October 19, 2000

Abstract Mizoribine, an immunosuppressive drug, has been used for treatment in organ transplantation, lupus nephritis, and rheumatoid arthritis (RA). On the basis of in vitro experiments, mizoribine has been postulated to be an inhibitor of inosine monophosphate (IMP) dehydrogenase, a pivotal enzyme in the formation of guanine ribonucleotides from IMP. To further characterize the mechanism of the antirheumatic action of this drug, we examined the effect of mizoribine on the production of interleukin (IL)-6, a major inflammatory cytokine in rheumatoid synovia, by freshly prepared rheumatoid synovial cells (RSC). Mizoribine (1.25–5 μg/ml) was able to inhibit the spontaneous production of IL-6 by fresh RSC in a dose–response fashion. The addition of guanosine monophosphate (GMP) reversed its inhibitory effects. In addition, mizoribine inhibited the enhanced production of IL-6 by the IL-1α and/or tumor necrosis factor α-stimulated RSC. Inhibition was also observed at the mRNA level, determined by Northern blot analysis. In contrast, mizoribine did not affect IL-8 production by these cells. These data suggest that mizoribine inhibits IL-6 production by fresh RSC, possibly owing to the depletion of intracellular GMP, and that this inhibitory effect of the drug on rheumatoid synovial cells may be related to its efficacy in RA.

Key words Guanosine triphosphate (GTP) · Interleukin-6 · mizoribine · Rheumatoid arthritis · Synovial cells

Introduction

Although the pathogenesis of rheumatoid arthritis (RA) is still unknown, a number of cytokines have been implicated as important mediators of inflammation and joint destruction.[1,2] Interleukin (IL)-6 is a pleiotropic cytokine that plays important roles in the inflammatory process in RA.[3] IL-6 induces the synthesis of acute-phase protein,[4] thrombocytosis,[5] and bone resorption.[6] IL-6 is produced by a variety of cell types, such as monocytes, fibroblasts, and endothelial cells. In particular, IL-6 has been shown to be the most abundantly expressed cytokine in rheumatoid synovia.[7,8] Moreover, recent studies demonstrated that an antibody against IL-6 receptor might be effective for the treatment of RA[9] and a murine collagen-induced arthritis model.[10]

Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazorium-5-olate) was isolated from the soil fungus *Eupenicillum brefeldianum.*[11] Mizoribine is metabolized to its monophosphate form by adenosine kinase,[12] and has been postulated to inhibit inosine monophosphate (IMP) dehydrogenase, which is required for the synthesis of guanine nucleotides from IMP.[13] It has been shown that mizoribine suppresses the proliferation of human T cells in a mechanism that is reversed by repletion of intracellular guanosine monophosphate (GMP).[14] Thus, mizoribine has an immunosuppressive action through inhibition of de novo synthesis of guanine nucleotides. In clinical conditions, mizoribine is widely used as an immunosuppressive drug for treatment in renal transplantation.[15,16] In addition, previous clinical trials demonstrated the efficacy of mizoribine in the treatment of RA[17] and lupus nephritis.[18]

Guanosine triphosphate (GTP) is required for many biological activities such as the synthesis of DNA, RNA, and protein, and cell signaling. It is well established that GTP-binding proteins have diverse roles as switches in cell growth, receptor activation, cytokine signaling, protein exocytosis, and changes in cell shape.[19,20] In view of the important roles of GTP, it is likely that mizoribine may modulate the production of various cytokines in rheumatoid synovia through the deletion of intracellular guanine nucleotides. However, little is known about the effect of mizoribine on the production of inflammatory cytokines. In the present study, we examined the effect of mizoribine on the production of IL-6 by freshly prepared rheumatoid synovial cells.

E. Sugiyama (✉) · M. Ikemoto · H. Taki · M. Maruyama · N. Yamashita · M. Kobayashi
First Department of Internal Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan
Tel. +81-76-434-2281; Fax +81-76-434-5025
e-mail: esugiyam@nsknet.or.jp

materials and methods

Reagents

Mizoribine was provided by Asahi Chemical Industry (Tokyo, Japan). Tumor necrosis factor α (TNFα) (recombinant human TNFα, specific activity 2.55×10^6 units/mg) and IL-1α (recombinant human IL-1α, specific activity 2.01×10^7 units/mg) were generous gifts from Dainippon Pharmaceutical (Osaka, Japan). These cytokines contained no detectable levels of endotoxin. GMP and dimethylthiozoldiphenyl tetrazolium bromide (MTT-tetrazolium) were purchased from Sigma Chemical (St Louis, MO, USA).

Cell preparation

The freshly prepared rheumatoid synovial cells (fresh RSC) used in this study were derived from patients with RA who underwent total knee replacement or synovectomy. All patients were diagnosed as having RA according to the 1987 criteria of the American College of Rheumatology.[21] Informed consent was obtained from each patient. The synovial tissue was cut into fragments of 1–3 mm diameter and incubated in 0.5–1 mg/ml collagenase and 5–10 μg/ml deoxyribonuclease I for 2–3 h. After digestion, the resultant single-cell suspension was washed, filtered through sterile gauze and nylon mesh, and finally resuspended in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin 100 U/ml, gentamicin 60 μg/ml, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid 12.5 mM, and L-glutamine 2 mM (DMEM medium). After overnight incubation, nonadherent cells were removed. The resultant adherent synovial cells were used as fresh RSC.

Immunoassay of IL-6 and IL-8

Fresh RSC were plated at a density of 1×10^6 cells /ml in 24-well plates. These cells were cultured in the presence or absence of IL-1α and TNFα for the duration indicated. The culture supernatants were collected, and stocked at −20°C until used. IL-6 and IL-8 concentrations in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's directions. The ELISA kits for both cytokines were purchased from Research & Diagnostic Systems (Minneapolis, MN, USA).

Cell viability

A colorimetric MTT incorporation assay was used as an indicator of cell viability.[22] MTT–tetrazolium was dissolved in PBS at 5 mg/ml. Twenty μl of a stock MTT solution was added to all wells, and the plates were further incubated for 1–2 h. The culture supernatant was then removed and 300 μl of dimethylsulfoxide was added to each well and mixed thoroughly. Once the MTT crystals were dissolved, triplicated aliquots (80 μl) of the supernatant were pipetted into 96-well plates. The absorbance was then read at $OD_{570-630}$ using a microplate reader.

RNA isolation and Northern blot analysis

Total cellular RNAs in the treated fresh RSC were extracted by acid guanidine phenol/chloroform extraction[23] using ISOGEN (Nippon Gene, Toyama, Japan). The total RNAs were fractionated in 1% agalose/3.7% formaldehyde gels for Northern blot analysis. rRNA was stained with ethidium bromide and visualized under ultraviolet light. The RNA was transferred to a nylon membrane (Hybond-N; Amersham, UK). An IL-6 cDNA probe was obtained after restriction enzyme digestion of total plasmid. The vector pGEM4, containing the cDNA of IL-6 as an insert of 440 bp, was a generous gift of Dr. Hirano (Osaka, Japan). The probe was labeled with [digoxigenin] dUTP by using a nonradioactive DNA labeling and detection kit (Boehringer Mannheim Mannheim, Germany). Prehybridization and hybridization in 50% formamide were carried out at 42°C. Final washes were performed in $0.1 \times$ SSC and 0.2% SDS at 65°C. The membranes were then incubated with antidigoxigenin alkaline phosphatase conjugate, washed, and incubated with a chemiluminescent substrate for alkaline phosphatase, adamantyl-1,2-dioxetane phosphate, before being exposed to X-ray film (Eastman Kodak, Rochester, NY, USA).

Results

Inhibitory effect of mizoribine on the production of IL-6 by fresh RSC

Fresh RSC spontaneously produced large amounts of IL-6 (Fig. 1A). Mizoribine inhibited this spontaneous production in a dose–response fashion (Fig. 1A). The inhibitory effect was observed at a concentration of 1.25 μg/ml mizoribine, which was almost the same as that reached in blood in vivo during mizoribine treatment.[24] The inhibitory effect of mizoribine was not due to the cell cytotoxicity of this drug, assessed by colorimetric MTT assay (Fig. 1B). As shown in Fig. 1C, the spontaneous production of IL-6 was rapidly reduced after 24 h, but the synovial cells looked alive under the microscope even after 72 h culture. In addition, we confirmed that the cell viability was always more than 95% by using a trypan blue dye exclusion test. Therefore, the decline of IL-6 production was due to the change in activation state, not to cell death. As shown in Fig. 1C, mizoribine inhibited the production of IL-6 in all time points except the first incubation of 0–12 h.

The role of GTP in IL-6 inhibition by mizoribine

We next examined whether the inhibitory effect of mizoribine might be via the deletion of intracellular guanine

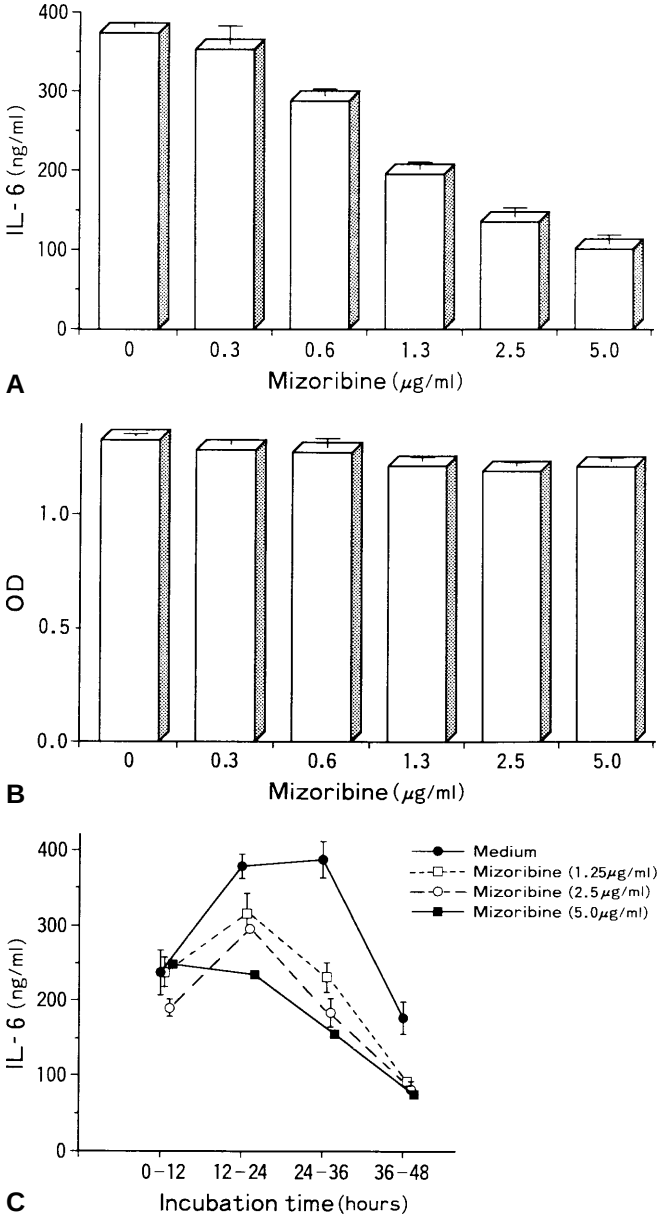

Fig. 1. Effect of mizoribine on the production of interleukin (IL)-6 by freshly prepared rheumatoid synovial cells (fresh RSC). **A** Fresh RSC were placed in a 24-well plate at 5×10^5 cells/well in 0.5 ml DMEM medium (see Materials and methods), and cultured in the presence of increasing concentrations of mizoribine every 12 h. The amounts of IL-6 in the supernatants of the culture medium from 24 to 36 h were measured by enzyme-linked immunoabsorbent assay (ELISA). **B** After incubation with mizoribine for 24 h, the viability of the cells was assessed by colorimetric MTT incorporation assay (see Methods). *OD*, optic density. **C** For time-course studies, fresh RSC were cultured with the indicated concentration of mizoribine, and the supernatants were replaced with fresh medium containing mizoribine every 12 h. The amounts of IL-6 in each supernatant were measured by ELISA. Each *bar* represents mean ± SD of three different wells. Data are representative of three separate experiments with rheumatoid synovia obtained from three different patients

nucleotides. The serum levels of mizoribine in patients with RA could reach a concentration of 2.5 μg/ml. To examine the clinical significance of this study, we tested the effect of mizoribine at a concentration of 2.5 μg/ml. As shown in Fig. 2, mizoribine inhibited IL-6 production by fresh RSC.

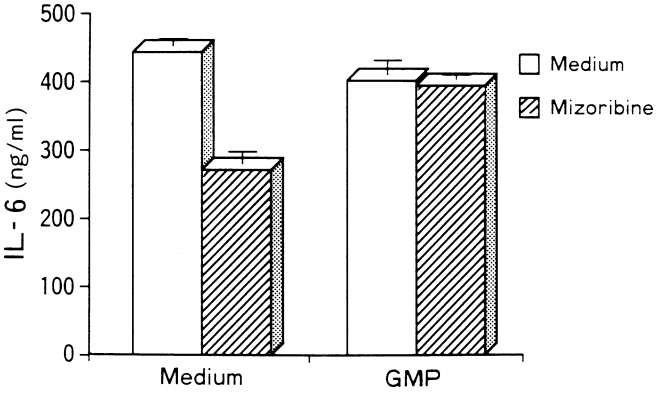

Fig. 2. Effect of guanosine monophosphate (GMP) on the inhibition of interleukin (IL)-6 production by mizoribine. Fresh RSC were placed in a 24-well plate at 5×10^5 cells/well in 0.5 ml DMEM medium, and cultured with or without mizoribine (2.5 μg/ml) in the presence or absence of GMP (40 μM) every 24 h. The amounts of IL-6 in the supernatants of culture medium from 24 to 48 h were measured by ELISA. Each *bar* represents mean ± SD of four different wells. Data are representative of three separate experiments with rheumatoid synovia obtained from three different patients

The addition of GMP to the culture completely abolished the inhibitory effect of mizoribine, suggesting that the IL-6 inhibition is mainly due to decreased levels of guanine nucleotides.

The effect of mizoribine on the production of IL-6 and IL-8 by IL-1α- and/or TNFα-stimulated RSC

It has been shown that IL-1 and TNFα, the major cytokines in rheumatoid synovia,[1,2] stimulate IL-6 production in a variety of cell types. Therefore, it is possible that mizoribine indirectly inhibited IL-6 production via inhibition of these cytokines. Next, we examined the effect of mizoribine on the production of IL-6 by fresh RSC cultured in the presence or absence of exogenous IL-1α and/or TNFα. The addition of both cytokines resulted in the increased production of IL-6 and IL-8 by RSC (Fig. 3A,B). Mizoribine also inhibited the enhanced production of IL-6 (Fig. 3A). In contrast, mizoribine did not modulate the production of IL-8 (Fig. 3B). We next examined the effect of mizoribine on the gene expression of IL-6. Northern blot analysis revealed that IL-1α induced the gene expression of IL-6, and that mizoribine also inhibited the enhanced expression of the gene (Fig. 3C).

Discussion

Mizoribine is widely used as an immunosuppressant for the treatment of RA in Japan.[17] Although some inhibitory effects of mizoribine on the proliferation of T cells[14] and B cells[25] have been reported, little is known about the effect of mizoribine on the rheumatoid synovial cells. We have demonstrated that mizoribine inhibited IL-6 production by fresh RSC. In total, more than 15 samples were analyzed for this study, and the inhibition of IL-6 by mizoribine was

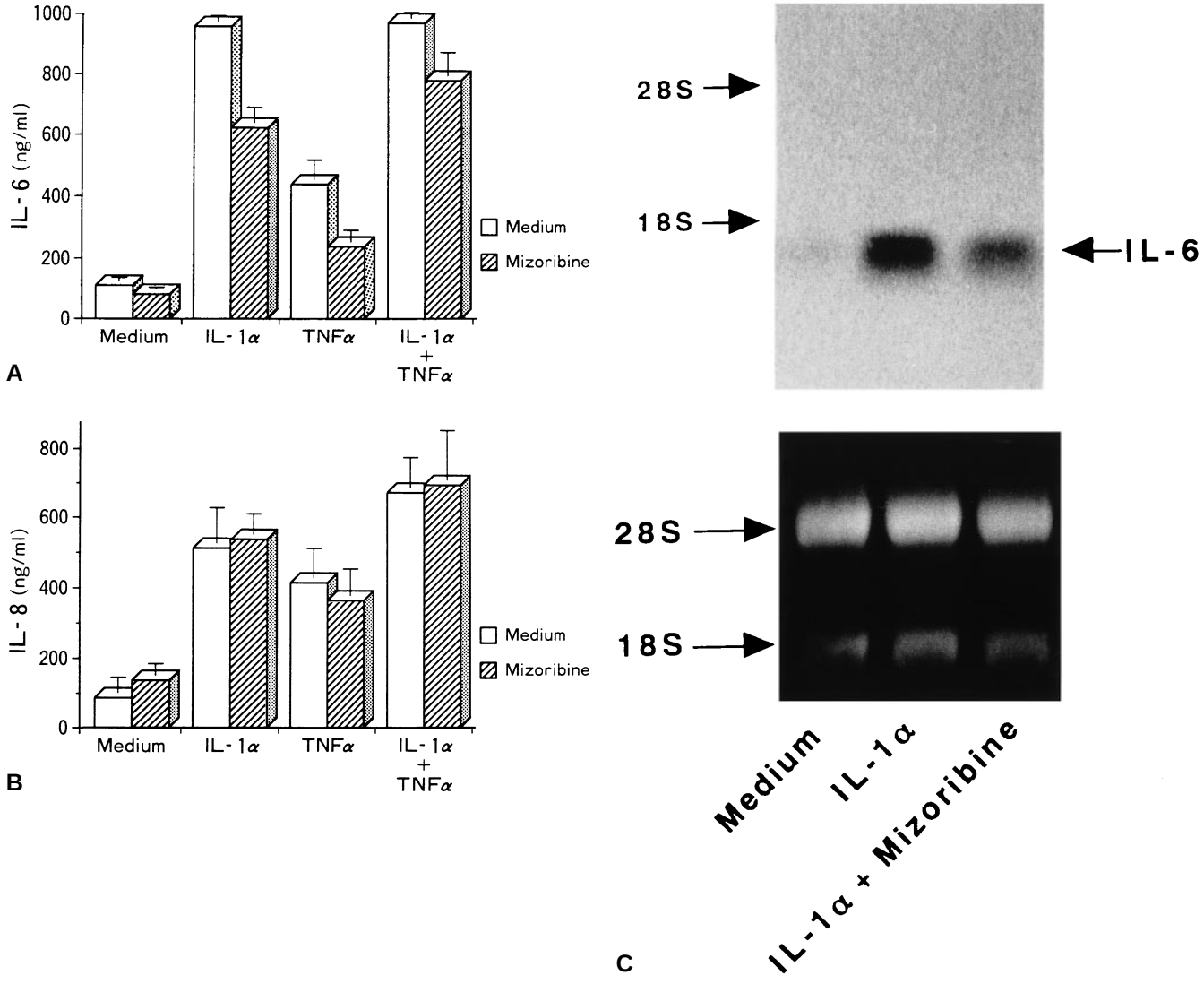

Fig. 3. Effect of mizoribine on the production of interleukin (IL)-6 and IL-8 by IL-1α and/or tumor necrosis factor α (TNFα). The rheumatoid synovial cells, after being cultured for 7 days after isolation, were placed in a 24-well plate at 5×10^5 cells /well, and cultured with or without IL-1α (2 ng/ml) and/or TNFα (20 ng/ml) in the presence of 2.5 μg/ml mizoribine for 24 h. The amounts of IL-6 (**A**) and IL-8 (**B**) in the culture supernatants were measured by ELISA. Each *bar* represents mean ± SD of three different wells. Data are representative of two separate experiments with rheumatoid synovia obtained from two different patients. **C** Northern blot analysis. Rheumatoid synovial cells after 7 days culture were cultured with or without IL-1α (2 ng/ml) in the presence or absence of mizoribine (5 μg/ml) for 8 h. After incubation, the treated cells were harvested for Northern blotting. Ten micrograms of total cellular RNA was size-fractionated, blotted, and hybridized with digoxigenin-labeled IL-6 probe (see Materials and methods). The amounts of rRNA stained with ethidium bromide are shown in the *lower panel*. Data are representative of two separate experiments with rheumatoid synovium obtained from two different patients

consistently observed in all experiments for time-course, dose–response, and the gene expression. In addition, the inhibition was completely prevented by exogenous GMP. To our knowledge, this is the first report examining the effect of mizoribine on the production of inflammatory cytokines by rheumatoid synovial cells.

Although the fresh RSC used in this study mainly consisted of macrophage-like cells and fibroblast-like cells,[26, 27] mizoribine did not affect IL-6 production by IL-1α-stimulated cultured rheumatoid synovial fibroblasts (data not shown). These contrasting data might be explained by the differences in intracellular guanine nucleotide levels after treatment with mizoribine, although we could not determine those levels. The GTP level in cells is regulated through de novo and salvage pathways. Although mizoribine inhibits de novo synthesis of GTP via inhibition of IMP dehydrogenase, it does not affect the salvage pathway. Therefore, it is likely that mizoribine may act mainly on the cells in which the de novo pathway of GTP synthesis is dominant. In fact, it has been demonstrated that mizoribine reduced the levels of GTP in T cells much more than those in B cells.[25] Thus, the inhibitory effect of mizoribine may be dependent on cell type and/or the state of activation.

The mechanism by which the deletion of guanine nucleotides induces the inhibition of IL-6 is not yet known. In addition to a general effect on DNA, RNA, and protein

synthesis, GTP plays an important role in the plasma membrane. GTP-binding protein participates in receptor-mediated signal transduction, including inflammatory mediators and proteinases.[19,20] It was reported that an activator of GTP-binding protein, sodium fluoride, could induce the expression of IL-6 mRNA in fibroblasts.[28] In addition, a recent study suggested that mizoribine might modulate the function of GTP-binding proteins in an apoptosis model using insulin-secreting cells.[29] Considering these findings, it is possible that GTP may be involved in excessive production of IL-6 in rheumatoid synovia, and that mizoribine may inhibit IL-6 production by modulating the function of GTP-binding proteins.

Mizoribine did not affect IL-8 production. In addition, IL-1β production was not inhibited by mizoribine (data not shown), although we could not examine the effect of mizoribine on the production of TNFα because the amounts of TNFα produced by the synovial cells were so small. These results indicated that the inhibitory effect of mizoribine might be relatively restricted to IL-6. The 5′ flanking region of the IL-6 gene contains a number of putative *cis*-acting elements which may be modulated by cyclic AMP.[30] In fact, the gene expression of IL-6 was up-regulated by cyclic AMP or prostaglandins.[31,32] Since GTP-binding proteins play a critical role in cyclic AMP-mediated signal transduction,[19,20] mizoribine may inhibit IL-6 gene expression by inhibition of the signal transduction.

In conclusion, we have shown that mizoribine inhibited IL-6 production by fresh RSC, possibly due to decreased levels of intracellular guanine nucleotides. The inhibitory effect of this drug may be related to its efficacy in the treatment of RA.

Acknowledgments The authors gratefully acknowledge the Asahi Chemical Industry Co. for providing mizoribine, Dainippon Pharmaceutical for providing recombinant human IL-1α and TNFα, and Drs. Hitoshi Yamada and Kouichi Kanekasu for rheumatoid synovial specimens.

References

1. Arend W, Dayer J-M. Cytokine and cytokine inhibitors or antagonists in rheumatoid arthritis. Arthritis Rheum 1990;33:305–15.
2. Arend W, Dayer J-M. Inhibition of the production and effects of interleukin-1 and tumor necrosis factor α in rheumatoid arthritis. Arthritis Rheum 1995;38:151–60.
3. Van Snick J. Interleukin 6: an overview. Ann Rev Immunol 1990;8:253–378.
4. Heinrich P, Castell J, Andus T. Interleukin 6 and the acute-phase response. Biochem J 1990;265:621–36.
5. Hill R, Warren M, Levin J. Stimulation of thrombopoiesis in mice by human recombinant interleukin 6. J Clin Invest 1990;85:1242–7.
6. Ishimi Y, Miyaura C, Jin C, Akatsu T, Abe E, Nakamura Y, et al. IL-6 is produced by osteoblasts and induces bone resorption. J Immunol 1990;145:3297–303.
7. Firestein G, Alvaro-Gracia JM, Maki R. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J Immunol 1990;144:3342–53.
8. Guerne PA, Zuraw BL, Vaughan JH, Carson DA, Lotz M. Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis. J Clin Invest 1989;83:585–92.
9. Yoshizaki K, Nishimoto N, Mihara M, Kishimoto T. Therapy of rheumatoid arthritis by blocking IL-6 signal transduction with a humanized anti-IL-6 receptor antibody. Springer Semin Immunopathol 1998;20:247–59.
10. Takagi N, Mihara M, Moriya Y, Nishimoto N, Yoshizaki K, Kishimoto T, et al. Blockage of interleukin-6 receptor ameliorates joint disease in murine collagen-induced arthritis. Arthritis Rheum 1998;41:2117–21.
11. Mizuno K, Tsujino M, Takada M, Hayashi M, Atsumi K. Studies on bredinin. I. Isolation, characterization and biological properties. J Antibiot 1974;27:775–82.
12. Koyama H, Tsuji M. Genetic and biochemical studies on the activation and cytotoxic mechanism of bredinin, a potent inhibitor of purine biosynthesis in mammalian cells. Biochem Pharmacol 1983;32:3547–53.
13. Sakaguchi K, Tsujino M, Mizuno K, Hayano K, Ishida N. Effect of bredinin and its aglycone on L5178Y cells. J Antibiot 1975;28:798–803.
14. Turka LA, Dayton J, Sinclair G, Thompson CB, Mitchell BS. Guanine ribonucleotide depletion inhibits T cell activation. Mechanism of action of the immunosuppressive drug mizoribine. J Clin Invest 1991;87:940–8.
15. Tajima A, Hata M, Ohta N, Ohtawara Y, Suzuki K, Aso Y. Bredinin treatment in clinical kidney allografting. Transplantation 1984;38:116–8.
16. Osakabe T, Uchida H, Masaki Y, Yokota K, Sato K, Nakayama Y, et al. Studies on immunosuppression with low-dose cyclosporine combined with mizoribine in experimental and clinical cadaveric renal allotransplantation. Transplant Proc 1989;21:1598–600.
17. Shiokawa Y, Homma M, Shichikawa K, Miyamoto T, Hirose S, Nobunaga T, et al. Clinical effectiveness of mizoribine on rheumatoid arthritis: a double-blind comparative study using lobenzarit disodium as a standard drug (in Japanese). Jpn J Inflamm 1991;11:375–96.
18. Homma M, Akizuki M, Yokohari R, Hashimoto H, Kashiwazaki S, Kondo H, et al. Clinical evaluation of mizoribine on lupus nephritis: multicenter single-blind comparative study with inactive placebo (in Japanese). Rinsho-iyaku 1989;5:795–824.
19. Watson S, Arkinstall S. The G-protein linked receptor facts book. London: Academic Press; 1994.
20. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. Annu Rev Biochem 1998;67:653–92.
21. Arnett F, Edworthy S, Bloch D, McShane D, Fries J, Cooper N, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
22. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. J Immunol Methods 1983;65:55–63.
23. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987;162:156–9.
24. Hosotsubo H, Takahara S, Taenaka N. Simplified high-performance liquid chromatographic method for determination of mizoribine in human serum. J Chromatogr 1988;432:340–5.
25. Hirohata S, Yanagida T. Inhibition of expression of cyclin A in human B cells by an immunosuppressant mizoribine. J Immunol 1995;155:5175–83.
26. Graabaek PM. Ultrastructural evidence for two distinct types of synoviocytes in rat synovial membrane. J Ultrastruct Res 1982;78:321–39.
27. Wilkinson LS, Pitsillides AA, Worrall JG, Edwards JC. Light microscopic characterization of the fibroblast-like synovial intimal cell (synoviocyte). Arthritis Rheum 1992;35:1179–84.
28. Akashi M, Loussararian AH, Adelman DC, Saito M, Koeffler HP. Role of lymphotoxin in expression of interleukin 6 in human fibroblasts. Stimulation and regulation. J Clin Invest 1990;85:121–9.
29. Li G, Segu VBG, Rabaglia ME, Luo R, Kowluru A, Metz SA. Prolonged deletion of guanosin triphosphate induces death of insulin-secreting cells by apoptosis. Endocrinology 1998;139:3752–62.
30. Ray A, Tatter SB, May LT, Sehgal PB. Activation of the human β2-interferon/hepatocyte-stimulating factor/interleukin-6 promoter by cytokines, viruses, and second messenger agonists. Proc Natl Acad Sci USA 1988;85:6701–5.

31. Bailly S, Ferrua B, Fay M, Gougerot-Pocidalo MA. Differential regulation of IL-6, IL-1α, IL-1β and TNFα production in LPS-stimulated human monocytes: role of cyclic AMP. Cytokine 1990;2:205–10.

32. Dendorfer U, Oettgen P, Libermann TA. Multiple regulatory elements in the interleukin-6 gene mediate induction by prostaglandins, cyclic AMP, and lipopolysaccharide. Mol Cell Biol 1994;14: 4443–54.