**Mizoribine Inhibits Production of Pro-inflammatory Cytokines and PGE2 in Macrophages**

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**ABSTRACT**

**Background:** Mizoribine (MZR) is an imidazole nucleoside isolated from *Euphorbiium hirtellum*. MZR is currently in clinical use for patients who have undergone renal transplantation. Therapeutic efficacy of MZR has also been demonstrated in rheumatoid arthritis and lupus nephritis. MZR has been shown to inhibit the proliferation of lymphocytes by interfering with inosine monophosphate dehydrogenase. Since the exact mechanism by which MZR benefits rheumatoid arthritis (RA) is not clear, we investigated the ability of MZR to direct its immunosuppressive influences on other antigen presenting cells, such as macrophages. **Methods:** Mouse macrophage RAW264.7 cells were stimulated with lipopolysaccharide in the presence of MZR. To elucidate the mechanism of the therapeutic efficacy in chronic inflammatory diseases, we examined the effects of MZR on the production of pro-inflammatory cytokines, nitric oxide (NO) and prostaglandin E₂ (PGE₂) in macrophages. **Results:** MZR dose-dependently decreased the production of nitric oxide and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukins 1β (IL-1β) and IL-6 PGE₂. Examination of gene expression levels showed that the anti-inflammatory effect correlated with the down-regulation of inducible nitric oxide synthase expression, cyclooxygenase-2 expression and TNF-α gene expression. **Conclusion:** In this work, we resulted whether MZR (1.25-10 μg/ml) inhibited macrophage activation by inhibiting secretion of pro-inflammatory cytokines, NO and PGE₂. These findings provide an explanation for the therapeutic efficacy of MZR in chronic inflammation-associated diseases. (Immune Network 2007;7(1):31-38)

**Key Words:** Mizoribine, anti-inflammation, macrophage, nitric oxide, IL-1β, TNF-α, IL-6

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**Introduction**

Macrophages play an important role in immune and allergic reactions, as well as in inflammation (1). In response to microbes and their products such as lipopolysaccharide (LPS), macrophages secrete various inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-12, and TNF-α through the activation of nuclear factor (NF)-κB; they also express NF-κB-dependent inducible nitric oxide (NO) synthase (iNOS) (2,3) and cyclooxygenase-2 (COX-2) (4). Many diseases, such as rheumatoid arthritis (RA), arteriosclerosis, chronic hepatitis, and pulmonary fibrosis (5-8), involve the overproduction of inflammatory mediators. Thus, inhibition of the production of these inflammatory mediators may prevent or suppress a variety of inflammatory diseases, including RA, arteriosclerosis, sepsis, and endotoxemia.

The exact causes of autoimmune disease remain obscure, but deregulated overproduction of pro-inflammatory cytokines or disruption in the regulation of cytokine signal transduction are underlying mechanisms of some autoimmune diseases such as RA and Crohn's disease (9,10). RA is characterized by synovial inflammation and the destruction of cartilage and bone (11). The pathology of RA extends throughout the synovial joint, and in severe cases involves many other organs. In contrast to the cellular nature of
normal synovial fluid, RA synovial fluid is enriched predominantly with neutrophils, but macrophages, T lymphocytes, and dendritic cells are also present (12). Macrophages and T lymphocytes are the most abundant cells in the synovial membrane, which also includes plasma cells, dendritic cells, and activated fibroblasts. Many of these cells are activated and express abundant HLA class II and adhesion molecules relevant to antigen presentation (13,14).

Mizorine (MZR) is an imidazole nucleoside isolated from Espenillium brasiliense (Fig. 1). MZR possesses immunosuppressive activity in that it selectively inhibits the proliferation of lymphocytes by interfering with inosine monophosphate dehydrogenase (15). The efficacy of this agent has been demonstrated not only in patients who have had renal transplantation, but also in patients with RA, lupus nephritis and primary nephritic syndrome. Recent studies have also shown that MZR suppresses the proliferation of human T and B cells (16). Recent clinical trials have demonstrated its efficacy in RA and lupus nephritis, in which B cells function abnormally. Since B cells are one type of antigen presenting cells (APCs), we investigated the ability of MZR to direct its immunosuppressive influences on other antigen presenting cells, such as macrophages. Since the exact mechanism by which MZR benefits RA patients is not clear, the purpose of this study was to determine whether MZR decreased the production of pro-inflammatory cytokines in macrophages.

### Materials and Methods

**Cell culture** The murine macrophage cell line (RAW 264.7) was purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, USA) and penicillin (100 U/ml)/streptomycin (100 U/ml).

**Cytokine and PGE2 assays** RAW 264.7 cells were cultured in 12-well flat plates at a density of 5 × 10^5 cells/well. The cells were treated with various concentrations (1.25, 2.5, 5, 10 μg/ml) of MZR (Chong Kun Dang Pharmaceutical Co. Seoul, Korea) and LPS (100 ng/ml) at 37°C for 48 hrs in humidified air with 5.5% CO₂. The supernatants were then collected and assayed for IL-1β, TNF-α IL-6 and PGE₂ content using IL-1β, TNF-α IL-6 and PGE₂ ELISA kits (BD Biosciences, USA).

**Nitric oxide assay** RAW 264.7 cells were added to each well (200 μl: 1 × 10^6 cells/ml) of flat-bottomed 96-well plates according to the following treatment conditions: LPS (50 ng/ml), LPS/MZR (1.25, 2.5, 5, 10 μg/ml), and media only (DMEM-10). NO production was determined according to the method reported by Stuehr and Nathan (17). Four wells were used for each group. The plates were incubated overnight, and 100 μl was then drawn from the surface of each well and transferred to a new plate. The transferred supernatant was mixed with an equal volume of Griess reagent (stock-I: 0.2% naphthylethendiamine HCl, stock-II: 2% sulfanilamide in 5% H₃PO₄) and incubated for 10 min at room temperature. NO production was measured by an ELISA reader at 540 nm. Standard calibration curves were prepared using sodium nitrite.

**Cell staining** To determine the effects of MZR on macrophage morphology, the cells were cultured in sterile glass-slide chambers at a density of 10,000 cells/well for 48 hrs. The culture medium was removed and the cells were treated with either LPS (100 ng/ml) or LPS/MZR (1.25, 2.5, 5, 10 μg/ml) for 2 days. Following treatment, the culture supernatant was removed. The cells were then fixed and stained in Diff-Quick Solution (Baxter, Houston, TX).

**RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction analysis (RT-PCR)** Total RNA was extracted from RAW264.7 cells using the RNasey Mini kit.
The RNA extraction was carried out in an RNAase-free environment. RNA was quantified by reading the absorbance at 260 nm according to the methods described by Majumder et al. (18). The reverse transcription of 1 μg RNA was carried out using M-MLV reverse transcriptase (Promega, USA), oligo (dT) 16 primer, dNTP (0.5 μM) and 1 U RNAse inhibitor. After incubation at 65°C for 5 min and 37°C for 60 min, M-MLV reverse transcriptase was inactivated by heating at 70°C for 15 min. The polymerase chain reaction (PCR) was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂ and 2.5 mM dNTPs with 5 units of Taq DNA polymerase and 10 μM of each primer set for IL-1β, TNF-α, IL-6, iNOS and COX-2. The amplification was followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min. Final extension was performed at 72°C for 5 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The nucleotides sequence of each primers and the size of product are shown in Table 1. β-actin was used as an internal control.

Western blot analysis. RAW 264.7 cells were washed with phosphate-buffered saline (PBS) and lysed by boiling in lysis buffer (1% SDS, 1.0 mM sodium vanadate, 10 mM Tris-Cl buffer, pH 7.4) for 5 min. 48 μg protein from the cell lysates was applied to 12% SDS-polyacrylamide gels and then transferred to nitrocellulose membrane. The membranes were blocked with a solution containing 5% BSA for 1 h. They were then incubated with anti-TNF-α, anti-IL-1β, anti-IL-6, anti-iNOS and anti-COX-2 monoclonal antibodies for 2 hrs and washed 3 times with PBS. After incubation with alkaline phosphatase-labeled secondary antibody for 2 hrs, the bands were visualized using Western Blot Kit substrate for phosphatase (LumiGLO System, KPL, USA).

### Results

**Effect of MZR on NO production in RAW 264.7 cells.** To examine the effects of MZR on NO production, RAW 264.7 cells were incubated with 50 ng/ml of LPS in the presence of various MZR concentrations (1.25, 2.5, 5, 10 μg/ml). After overnight culture, the supernatants were collected, and the accumulated nitrite concentration was measured using Griess reagent. The macrophages did not release NO in response to medium alone (Fig. 2). LPS was used as a positive control for macrophage activation. In LPS (50 ng/ml)-stimulated RAW 264.7 cells, MZR increased NO production. When various concentrations of MZR (1.25, 2.5, 5, 10 μg/ml) were added to the culture media at the time of LPS-stimulation, NO production was decreased in a MZR concentration-dependent manner. To examine whether the inhibitory effects of MZR on NO production were attributable to its influence on the expression of mRNAs, semi-quantitative RT-PCR experiments were performed for the iNOS mRNA. As shown in Fig. 2B, the transcripts for iNOS were barely detectable in unstimulated RAW cells. The transcripts of iNOS transcripts, however, were readily detectable when RAW264.7 cells were stimulated with LPS. When LPS and MZR were added together, the amounts of iNOS transcripts were decreased significantly in RAW264.7 cells. Western blot analysis also confirmed that MZR inhibits the expression level of iNOS (Fig. 2C).

**Direct cytokines production in response to MZR.** To determine whether MZR (1.25, 2.5, 5, 10 μg/ml) had a direct effect on cytokine production, TNF-α, IL-1β and IL-6 secretion were measured in the macrophage cell line using cytokine ELISA kits. TNF-α, IL-1β and IL-6 are the major proinflammatory cytokines that are produced by monocytes and macrophages. As shown in Fig. 3A, 4A and 5A MZR decreased cytokines production in the presence of LPS (100

| Table I. The sequences of primers used in RT-PCR analysis |
|-------------|-----------------|
| Gene | Primer sequences |
| IL-1β | F 5'-CAGGAATGGACATGACACC-3' |
| R 5'-CTCTGAGACTCAATCTCCAC-3' |
| iNOS | F 5'-AGCTCTTCCAGGACCACAC-3' |
| R 5'-AGCGTGGATCTCTATGCCG-3' |
| TNF-α | F 5'-TGGACTTCGCGCTAGTG-3' |
| R 5'-CTCTGAGCCACGTCGAGC-3' |
| COX-2 | F 5'-AAGAAGAAGTTCATCTCAATCCC-3' |
| R 5'-TGACTTGAGGATACATCTTC-3' |
| IL-6 | F 5'-GTACCCAGAAGACCAGAG-3' |
| R 5'-TCGCTGTCGACACCCAGCC-3' |
| β-actin | F 5'-GTGGGCGCGCTAGGACCAG-3' |
| R 5'-GGAGGAAGAGATGCGGAGT-3' |
Figure 2. Effects of MZR on NO production in LPS (lipopolysaccharide)-stimulated RAW 264.7 cells. (A) The cultures were incubated with 50 ng/ml of LPS in the presence of various MZR concentrations (1.25, 2.5, 5, 10 μg/ml). 4 wells were used per group, and 200 μl (1 × 10⁶ cells/ml) of the cells were added to each well. The plates were then incubated overnight, and 100 μl from the surface of each well was transferred to a new plate. The accumulated nitrite concentration was measured after stimulation overnight using Griess reagent for 10 min at room temperature and read using an ELISA reader at 540 nm. NO production was determined by the accumulation of nitrite using the method reported Stuehr and Nathan (1989). (B) Levels of iNOS mRNA in cells stimulated with LPS for 24 h were determined by RT-PCR analysis. β-actin was used as internal control. (C) Levels of iNOS protein in cells stimulated with LPS for 48 h were measured by Western blot analysis using a monoclonal antibody for murine iNOS.

Figure 3. Effects of MZR on TNF-α production and TNF-α gene expression. RAW 264.7 cells (5 × 10⁵ cells/ml) were cultured with the indicated MZR for 48 h. (A) The TNF-α cytokine level was measured by TNF-α ELISA. (B) Levels of TNF-α mRNA in cells stimulated with LPS for 24 h were determined by RT-PCR analysis. β-actin was used as internal control. (C) Levels of TNF-α protein in cells stimulated with LPS for 48 h were measured by Western blot analysis using a monoclonal antibody for murine TNF-α.

ng/ml) in a dose-dependent manner. Interestingly, MZR strongly inhibited TNF-α production. We next examined whether MZR dose-dependently suppressed the mRNA levels of the proinflammatory cytokines in LPS-stimulated cells by RT-PCR analysis (Fig 3B, 4B and 5B). We also determined the intracellular levels of the same cytokines by Western blot analysis, again showing that MZR decreased the cellular levels of TNF-α, IL-1β and IL-6 induced by LPS in a dose-dependent manner (Fig 3C, 4C and 5C).

PG E₂ production. The PGE₂ production in macrophages was determined in the presence of MZR (1.25, 2.5, 5, 10 μg/ml) in both the presence and absence of LPS. PGE₂ production was measured in the macrophage cell line using PGE₂ ELISA kits. As shown in Fig. 6A, MZR decreased PGE₂ production...
in the presence of LPS (100 ng/ml) with a dose-dependent manner. *Expression of COX-2*: MZR dose-dependently suppressed the mRNA levels of COX-2 in LPS-stimulated cells by RT-PCR analysis (Fig. 6B). We also determined the intracellular levels of COX-2 by Western blot analysis, again showing that MZR decreased the cellular levels of COX-2 induced by LPS in a dose-dependent manner (Fig. 6C). *Effect of MZR and LPS on macrophage morphology*: Normal RAW 264.7 cells, when cultured in medium alone, tend to be round, with none appearing to spread over the surface (Fig. 7A). Fig. 7 shows that the cells treated with LPS alone (100 ng/ml; Fig. 7B) were larger and rougher than those exposed to 5 µg/ml (Fig. 7E) or 10 µg/ml (Fig. 7F). These results suggest that cells treated with LPS and low concentrations of MZR (1.25~2.5 µg/ml) were similar those exposed to LPS alone. However, cells treated with high concentrations of MZR (5~10 µg/ml) in combination with LPS...
Figure 6. Effects of MZR on PGE2 production and COX-2 gene expression. RAW 264.7 cells (5×10^5 cells/ml) were cultured with the indicated MZR for 48 h. (A) The PGE2 cytokine levels were measured in the PGE2 ELISA. (B) Levels of COX-2 mRNA in cells stimulated with LPS for 24 h were determined by RT-PCR analysis. β-actin was used as internal control. (C) Levels of COX-2 protein in cells stimulated with LPS for 48 h were measured by Western blot analysis using a monoclonal antibody for murine COX-2.

Figure 7. Morphological changes in macrophages in response to MZR. RAW 264.7 cells were cultured on cover slips in the presence of various concentrations of LPS/MZR in the medium: medium alone (A), LPS (100 ng/ml; B), LPS/MZR (1.25 μg/ml; C), LPS/MZR (2.5 μg/ml; D), LPS/MZR (5 μg/ml; E), and LPS/MZR (10 μg/ml; F) for 48 h. The cells were fixed and stained in Diff-quick Solution. The cells in MZR treated groups were smoother than those treated with LPS alone.

Discussion
RA is an autoimmune disease characterized by synovial inflammation and the destruction of cartilage and bone. For the past 20 years, MZR has been used treat patients with renal transplants and lupus nephritis (19-21). MZR suppresses the proliferation of synovial
fibroblasts and offers clinical advantages to patients with RA (22). In the inflamed synovial tissue of patients with RA, macrophages are the dominant cell population in areas adjacent to the cartilage junction, and the secretion of various cytokines from these cells, along with cellular invasion and degradation of the cartilage, plays a critical role in the process of inflammation (11).

Cytokines are local protein mediators that are now known to be involved in almost all important biological processes, including cell growth and activation, inflammation, immunity, and differentiation. Thus, it is not surprising that they have a role in an autoimmune disease such as RA, in which there is chronic inflammation with fibrosis and the eventual destruction of cartilage and bone (12). Pro-inflammatory cytokines, IL-1β and TNF-α, which are present in large quantities in inflammatory tissues, are continuously produced and responsible for aggravating the inflammation (23). These inflammatory cytokines are more abundant than Th2 cytokines, such as IL-4 and IL-13, which are also detectable in RA synovium and are anti-inflammatory cytokines. IL-4 can suppress the detrimental effects of IL-1 and TNF-α by stimulating IL-1 receptor antagonist (IL-1ra) production (24) and down-regulating the TNF receptor, transcription of metalloproteinase genes and synovial cell proliferation (25-27). Therefore, therapy for inflammatory diseases could be achieved either by interference with the action of pro-inflammatory cytokines or by supplementation of anti-inflammatory cytokines or cytokine inhibitors. The investigation in this study of the ability of MZR to direct immunosuppressive influences on antigen presenting cells, such as macrophages, demonstrated that MZR decreased the production of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 in macrophages. This anti-inflammatory effect occurs by the down-regulation of pro-inflammatory cytokine gene expression.

NO is an important part of the defense mechanism against infection, but when the response to the infection is excessive, superfluous NO damages normal cells and tissues. NO is often overproduced by activated macrophages. The activation of macrophages leads to iNOS expression and the production of NO through an intracellular reaction with L-Arg. LPS is known to enhance iNOS promoter activity and its expression (28). In the experiments included in the present study, macrophages were exposed to MZR for 12~48 h in the presence of LPS. Our results indicate that the inhibitory activity of MZR on macrophage activation is initiated immediately after exposure to these materials. In this work, we tested whether MZR (1.25~10 μg/ml) inhibited macrophage activation by inhibiting secretion of NO. We also examined the morphological changes that took place in the macrophages when treated with MZR and LPS.

In summary, the anti-inflammatory effect of MZR occurs through suppression of TNF-α, IL-1β, IL-6, iNOS and COX-2 gene expression.

Footnotes
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