Effect of Mizoribine on Effector T Cell-Mediated Immune Responses in Mice

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T cells play a principal role in cellular immunity and govern the regulatory mechanism in humoral immunity. Therefore, T cells play a key role as either effectors or regulators in the immune network. Mizoribine (MZR), an immunosuppressive agent, suppresses both humoral and cellular immunity by acting on both T cells and B cells. In this study, we examined the effect of MZR on various effector T cell-mediated immune responses in mice. MZR prolonged skin graft survival and suppressed a localized graft-versus-host reaction (GvHR) and sheep red blood cell (SRBC)-induced delayed-type hypersensitivity (DTH) reaction. In a collagen-induced arthritic mice model, MZR reduced the arthritic index and the swelling of the hind limbs. Furthermore, MZR suppressed both bone damage and histopathological changes in the hind limbs. Interestingly, MZR markedly suppressed the DTH reaction to type II collagen (CII) but had no effect on anti-CII antibody levels in this arthritic model. In these models, effector T cells such as cytotoxic T lymphocyte (CTL) and T_{DTH} cell play an important part in the development of these reactions. It is suggested that MZR inhibited these reactions *via* the inhibition of the effector T cell-mediated immune response. Therefore, it is also suggested that the suppressive effect of MZR on clinical rejection and autoimmune disease is based on its suppression of the effector T cell-mediated immune response, that is cellular immunity, in addition to humoral immunity.

Key words mizoribine; cytotoxic T lymphocyte; T_{DTH}; cellular immunity

Mizoribine (MZR) has generally been used for clinical kidney allograft recipients^{1–3)} and has recently been used to treat lupus nephritis⁴⁾ and rheumatoid arthritis.⁵⁾ MZR suppresses humoral and cellular immunity by the inhibition of the growth of both T cells and B cells^{6–11)} *via* the competitive inhibition of both inosine 5'-monophosphate (IMP) dehydrogenase and guanosine 5'-monophosphate (GMP) synthetase in the purine metabolism pathway, resulting in the depletion of intracellular GMP.^{12,13)}

Cellular immunity may play a principal role in the rejection of grafts and tumors, graft-versus-host disease (GvHD) and organ-specific autoimmune diseases. T cells, particularly effector T cells, are the most important cells involved in cellular immunity among the various types of immunocompetent cells which take part in the development of cellular immunity.

In the cellular immune response, MZR suppresses the blastogenic response of peripheral blood lymphocytes by mixed lymphocyte reaction (MLR).¹⁰⁾ MZR suppresses both picryl chloride- and purified protein derivative (PPD)-induced delayed-type hypersensitivity (DTH) reactions.^{7,14)} Furthermore, MZR inhibits a systemic graft-versus-host reaction (GvHR).¹⁴⁾ As mentioned above, the suppressive effect of MZR on several cellular immune responses was already examined. However, systematic examination focused on the effect of MZR on cellular immunity is not found.

To further clarify the effect of MZR on cellular immunity, we investigated the effect of MZR on various effector T cell-mediated immune responses. In this study, we chose three classic models in mice which are mediated mainly by cytotoxic T lymphocytes (CTLs) or T_{DTH} cells. In collagen-induced arthritis (CIA), which is an animal

model of rheumatoid arthritis, the induction and perpetuation of arthritis require synergy between humoral and cellular immunity.¹⁵⁾ It was shown that MZR has a suppressive effect on CIA in mice.¹⁶⁾ In this study, we also examined the effect of MZR at lower doses on this arthritis model and analyzed its effect on histopathological changes in arthritic paws.

MATERIALS AND METHODS

Animals Male C57BL/6, C3H/He, DBA/1J, BDF₁ and ddY mice were obtained from Japan SLC, Inc., Shizuoka, Japan. All mice were 7 to 8 weeks old when used. Animals were housed in plastic cages in an air-conditioned room at 22±1 °C, fed a standard laboratory diet and given water *ad libitum*.

Drugs MZR (kindly donated from Asahi Chemical Industry, Osaka, Japan) was dissolved in saline. Cyclosporin A (CsA; kindly donated from Sandoz Ltd., Basel, Switzerland) was suspended in 0.1% HCO-60 saline. Prednisolone acetate (Pred; Takeda Chemical Industries, Ltd., Osaka, Japan) was suspended in saline.

Skin Grafting Skin hair was removed by Eba cream (Tokyo Tanabe Co., Ltd., Tokyo, Japan) on the day before transplantation. The skin (radius of 5 mm) of C3H/He mice was grafted on the back of C57BL/6 mice. Recipient mice were dressed with gauze for 5 d. Necrosis of 50% of the surface was considered the time of acute rejection. Drugs were administered intraperitoneally (i.p.) twice a day, starting from the day of transplantation.

Localized GvHR Spleens were sterilely removed from C57BL/6 and BDF₁ mice, and single cell suspensions were prepared through a stainless steel sieve (200 mesh) in Hanks' balanced salt solution (HBSS) containing

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5 U/ml of heparin. After hemolysis by treatment with Tris-buffered ammonium chloride, splenocytes were washed three times with HBSS. The viability of the cells was evaluated using trypan blue dye exclusion.

Localized GvHR was induced by a modification of the method of Ford *et al.*¹⁷⁾ Briefly, 5×10^6 splenocytes of C57BL/6 mice in a volume of 50 μ l were injected subcutaneously (s.c.) into the right hind footpad, and the same amount of BDF₁ splenocytes was injected s.c. into the opposite footpad of BDF₁ mice. Eight days later, the recipients were sacrificed and popliteal lymph nodes (PLN) were removed from both extremities. The intensity of GvHR was expressed as the difference in weight between the right and left PLN. Drugs were administered i.p. for 8 d after the injection of splenocytes.

Sheep Red Blood Cell (SRBC)-Induced DTH Reaction

Hypersensitivity was measured by a modification of the method of Lagrange *et al.*¹⁸⁾ Briefly, male ddY mice were immunized s.c. with 1×10^7 washed SRBC (Toyo Bio Co. Ltd., Tokyo, Japan) in a volume of 40 μ l into the left hind footpad. Five days later, 1×10^8 SRBC in a volume of 40 μ l were inoculated into the right hind footpad, and after 24 h, the footpad volumes were measured using a plethysmometer (TK-101, Unicom, Chiba, Japan). The difference between the right and the left footpad was expressed in terms of intensity of the footpad reaction. Drugs were administered orally (*p.o.*) for 5 d after sensitization.

Type II Collagen-Induced Arthritis (CIA) in Mice Bovine type II collagen (CII; Cosmo Bio, Tokyo, Japan) dissolved in 0.01 M acetic acid was emulsified with an equal volume of complete Freund adjuvant (CFA; Difco Laboratories, MI, U.S.A.). CIA was induced in male DBA/1J mice by a modification of the method of Courtenay *et al.*¹⁹⁾ Briefly, an emulsion containing 200 μ g of CII was injected intradermally (i.d.) on the back skin. Three weeks after the initial injection, the same dose of CII emulsion was injected i.d. at the base of the tail. MZR was administered s.c. 6 d a week and Pred was administered 3 d a week for 9 weeks beginning on the day after the primary immunization.

The degree of arthritis in the metacarpophalangeal wrist, metatarsophalangeal, and ankle joints was scored as follows: 0, no arthritis; 1, slight arthritis; 2, light swelling; 3, medium swelling; 4, severe arthritis; 5, severe swelling and non-weight-bearing. The joint score was the sum of the scores of all involved joints. In addition, the paw volume was measured using a plethysmometer. At the end of the experimental period (9 weeks), both hind limbs were immersed in 10% buffered neutral formalin solution. Paraffin sections were prepared according to the routine method and histopathological examination was conducted. Histological lesions observed in the limb joints were graded in integers from 0 to 3. Radiographic evaluation was performed with a Softex (PK-5, Shimadzu Scientific Instruments, Ltd., Kyoto, Japan). The severity of arthritis in each paw, assessed by the extent of joint space narrowing and bone destruction, was graded in integers from 0 to 2. The radiographic joint index represents the sum of the hindpaw scores for each mouse. The cell-mediated immune response to CII was estimated

as follows: the thickness of both ears of each mouse was measured with a dial thickness gauge (R2-1A, Ozaki Seisakusho, Tokyo, Japan). Ten μ g of CII in phosphate buffered saline were injected into each ear, and the ear thickness of each animal was measured after 24 h to determine the intensity of *in vivo* cell-mediated immunity (DTH) to CII.

Statistical Analysis All data were represented as the mean $\pm$ S.E. and analyzed by Student's *t*-test and Dunnett's multiple range test. *p* values of less than 0.05 were considered to be statistically significant.

RESULTS

Suppressive Effect on Graft Rejection in Model of Allogeneic Grafting

Figure 1 shows the suppressive effect of MZR and CsA on allograft rejection. In skin-grafted mice, MZR significantly prolonged graft survival. Graft survival was 7.9 ± 0.2 d in the control group, 10.0 ± 0.6 d in the MZR 50 mg/kg $\cdot$ d group and 9.8 ± 0.5 d in the MZR 100 mg/kg $\cdot$ d group. CsA at doses of more than 20 mg/kg $\cdot$ d also significantly prolonged skin graft survival, and its effect was remarkable at a dose of 50 mg/kg $\cdot$ d.

Suppressive Effect on Localized GvHR in Mice The effect of MZR on localized GvHR was examined by intraperitoneal administration of the drug to BDF₁ mice for 8 d after the injection of splenocytes from C57BL/6 mice into the footpads. As shown in Fig. 2, MZR at doses of more than 20 mg/kg significantly suppressed the enlargement of PLN from the injection site of allogeneic cells, the enlargement of it being half that of the control group. Pred also inhibited GvHR, and this drug at a dose of 5 mg/kg showed almost equal activity with MZR at a dose of 50 mg/kg.

Suppressive Effect on SRBC-Induced DTH Reaction in Mice As shown in Fig. 3, MZR significantly suppressed the DTH reaction in a dose-dependent fashion at doses of more than 20 mg/kg when it was administered for 5 d after the sensitization. Pred also markedly suppressed the DTH reaction.

Fig. 1. Effect of MZR and CsA on Skin Graft Survival in Mice

C3H/He mice were used as donors and C57BL/6 mice were used as recipients. Donor skin grafts (radius of 5 mm) were sutured into the recipient flanks. Drugs were given intraperitoneally twice a day following the skin transplantation. The values represent the mean $\pm$ S.E. of 4–8 mice. ***p* < 0.01, significantly different from the control.

Fig. 2. Effect of MZR on Localized GvHR in Mice

GvHR was induced in BDF₁ mice by the subcutaneous injection of 5×10^6 splenocytes of C57BL/6 mice into the hind limb footpads. Drugs were given intraperitoneally for 8 successive days following the injection of splenocytes. Eight days later, popliteal lymph nodes were removed and their wet weights were measured. The values represent the mean $\pm$ S.E. of 5–7 mice. * $p < 0.05$, significantly different from the control.

Fig. 3. Effect of MZR on SRBC-Induced DTH Reaction in Mice

Male ddY mice were injected subcutaneously with 1×10^7 SRBC. Five days later, the mice were challenged by an injection of 1×10^8 SRBC into the right hind footpads. Drugs were given orally for 5 successive days following the sensitization. The volumes of both hind footpads were measured using a plethysmometer at 24 h after the challenge. The difference between the right and the left footpad volume was regarded as the intensity of the DTH reaction. The values represent the mean $\pm$ S.E. of 8 mice. * $p < 0.05$, ** $p < 0.01$, significantly different from the control.

Fig. 4. Effect of MZR on Type II Collagen-Induced Arthritis in Mice

DBA/1J mice were immunized with 200 μ g of type II collagen in CFA on days 0 and 21. The mice were treated subcutaneously with saline (○), MZR 5 mg/kg (●), MZR 10 mg/kg (□), and Pred 5 mg/kg (■) for 9 weeks beginning on the day after the primary immunization. The degree of arthritis in each mouse was scored on a scale of 0–5 (panel A) and footpad volume was measured using a plethysmometer once a week (panel B). The values represent the mean $\pm$ S.E. of 6–7 mice. ** $p < 0.01$, significantly different from the saline-treated control.

Suppressive Effect on CIA in Mice The effect of MZR on CIA was examined by the administration of the drug 6 d a week for 9 weeks to DBA/1J mice immunized twice with 200 μ g of CII in CFA. The mice developed arthritis after receiving the booster injection, and the arthritic index of the control group was 3.8 at 9 weeks after primary immunization (Fig. 4A). MZR significantly reduced the arthritic index, to 0.8 by 5 mg/kg and to 1.3 by 10 mg/kg, 9 weeks after the immunization. Pred at a dose of 5 mg/kg almost completely suppressed arthritis. At the same time, we measured the volume of the hind footpads. Both MZR and Pred also significantly suppressed an increase in footpad volume in addition to the arthritic index (Fig. 4B).

The bone damage scores of the hind limbs are shown in Fig. 5. In the control group, joint space narrowing and bone destruction were observed in all mice. MZR significantly suppressed these bone alterations, and Pred also

markedly suppressed them. Histopathological changes in the hind limb joints are shown in Table 1. Arthritic changes in the limbs were observed in all mice of the control group. MZR suppressed these changes, in particular, the degradation and erosion of cartilage and the proliferation of synovium were significantly suppressed. Pred almost completely suppressed the histopathological changes.

DTH reactions to CII at 9 weeks after primary immunization are shown in Fig. 6. MZR significantly suppressed the DTH reaction in a dose-dependent fashion, and Pred also markedly suppressed this reaction.

DISCUSSION

In this study, we focused on cellular immunity and investigated the effect of MZR on various effector T cell-mediated immune responses in animal model.

Fig. 5. Effect of MZR on Radiographic Index of Hind Limb Joints in Type II Collagen-Induced Arthritic Mice

DBA/1J mice were immunized with 200 μ g of type II collagen in CFA on days 0 and 21. At the end of the experimental period, both hind limbs were cut off. Soft X-ray photographs of the hind limbs were taken and the severity of arthritis was scored. The horizontal bar represents the mean value of 6–7 mice. ** p < 0.01, significantly different from the control.

Table 1. Effect of MZR on Histopathological Index of Hind Limb Joints in Type II Collagen-Induced Arthritic Mice

	Control	MZR		Pred	
		5	10	5	(mg/kg)
Degradation of cartilage	2.33 $\pm$ 0.33	0.71 $\pm$ 0.48	0.83 $\pm$ 0.17*	0.00 $\pm$ 0.00**	
Formation of pannus	1.67 $\pm$ 0.62	0.57 $\pm$ 0.37	1.33 $\pm$ 0.62	0.00 $\pm$ 0.00	
Proliferation of synovium	2.33 $\pm$ 0.33	0.86 $\pm$ 0.40*	1.00 $\pm$ 0.00*	0.00 $\pm$ 0.00*	
Erosion of cartilage	2.50 $\pm$ 0.22	0.86 $\pm$ 0.46*	1.00 $\pm$ 0.00**	0.33 $\pm$ 0.21	

DBA/1J mice were immunized with 200 μ g of type II collagen in CFA on days 0 and 21. At the end of the experimental period, both hind limbs were cut off. After the hind limbs were immersed in 10% buffered neutral formalin solution, the joints were decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The values represent the mean $\pm$ S.E. of 6–7 mice. * p < 0.05, ** p < 0.01, significantly different from the control.

Fig. 6. Effect of MZR on DTH Reaction to Type II Collagen in Type II Collagen-Induced Arthritic Mice

DBA/1J mice were immunized with 200 μ g of type II collagen in CFA on days 0 and 21. The mice were injected with 10 μ g of type II collagen in PBS into both ears at 9 weeks after primary immunization. After 24 h, the thickness of the ears was measured. The values represent the mean $\pm$ S.E. of 6–7 mice. ** p < 0.01, significantly different from the control.

MZR prolonged skin allograft survival, that is, it inhibited the rejection of an allograft. Concerning allogeneic grafting in animals, MZR has been also reported to prolong the graft survival of thyroid, liver, pancreas

and kidney transplantation.^{20–22}) Furthermore, MZR inhibited localized GvHR, showing significant inhibition at doses of more than 20 mg/kg. It was reported that MZR inhibited systemic GvHR in a dose-dependent fashion but slightly inhibited it at a dose of 25 mg/kg.¹⁴)

Acute allograft rejection is characterized by the necrosis of parenchymal cells and is usually accompanied by lymphocyte and macrophage infiltrates. These infiltrating leukocytes are responsible for the lysis of the graft parenchymal cells. Several different effector mechanisms may be involved in acute cellular rejection, including CTL-mediated lysis, activated macrophage-mediated lysis (as in DTH), and natural killer (NK) cell-mediated lysis. Several lines of evidence suggest that the recognition and lysis of foreign cells by alloreactive CD8⁺ CTLs is probably the most important mechanism of acute cellular rejection.²³) GvHR can injure the host and cause GvHD. GvHD is the principal limitation regarding the use of bone marrow transplantation. In animal models, acute GvHD is initiated by mature T cells present in the bone marrow inoculum. The elimination of mature donor T cells from the graft can prevent the development of GvHD.²³) Acute GvHR occurs when CTLs are induced by the action of helper T cells. According to the above results, MZR inhibited these T cell-mediated immune responses in which CTLs may mainly play a key role as effector

cells.

MZR significantly suppressed the SRBC-induced DTH reaction in a dose-dependent fashion when it was administered for 5 successive days after the sensitization. It has been reported that MZR suppresses the picryl chloride-induced DTH reaction in mice.¹⁴⁾ In a tuberculin reaction, MZR does not suppress hypersensitivity when it is administered at the induction phase. However, the administration of MZR at the time when DTH is induced with a purified protein derivative (PPD) results in the suppression of an already established sensitization.⁷⁾

In the DTH reaction, antigen-activated T cells (usually CD4⁺ Th1 cells; T_{DTH} cells) secrete cytokines, which have several effects. Some cytokines activate venular endothelial cells to recruit monocytes from the blood at the site of antigen challenge. Other cytokines convert the monocytes into activated macrophages that serve to eliminate the antigen. By means of cytokine secretion, T_{DTH} cells stimulate the function and focus the activity of nonspecific effector cells of natural immunity, thereby converting these cells into agents of specific immunity. Thus, the DTH reaction is a form of cellular immune reaction mediated by T_{DTH} cells.

MZR significantly reduced the arthritic index and suppressed the bone damage of CIA in mice at 5 mg/kg and 10 mg/kg. In this study, we found that MZR suppressed histopathological changes in the hind limbs. Before, we found that MZR dose-dependently reduced arthritis in mice at doses of more than 10 mg/kg.¹⁶⁾ We reconfirmed the suppressive effect of MZR on the arthritis, even at a dose of 5 mg/kg.

It was shown that the levels of both cellular and humoral immunity to CII rose at the time arthritis appeared.²⁴⁾ As this arthritis can be passively transferred by sera from donors immunized with CII, anti-CII antibodies function in the initiation of arthritis.^{25,26)} However, this antibody-mediated arthritis is transient and the histopathology of this arthritis is somewhat different from that observed in collagen-induced arthritis.²⁷⁾ On the other hand, the inoculation of a T cell line reactive to CII resulted in arthritis, albeit at a low incidence.²⁸⁾ Therefore, it is suggested that cellular immunity to CII is required, in addition to humoral immunity, for the perpetuated development of CIA.^{26,29)} Thus, we examined the effect of MZR on the immunological parameters in CIA. MZR markedly suppressed the DTH reaction to CII. However, MZR didn't affect anti-CII antibody levels at a dose of 10 mg/kg (data not shown). Considering the above results, we suggested that MZR suppressed T_{DTH}-mediated immune responses in CIA mice and also the SRBC-induced DTH reaction. It has been reported that MZR suppresses humoral immunity *in vivo* and *in vitro*.⁶⁻⁹⁾ However, in this model, MZR suppressed cellular immunity rather than humoral immunity. Therefore, it is suggested that MZR suppressed arthritis in these animals because of its effect on cellular immunity.

In conclusion, we have demonstrated that MZR has a suppressive effect on CTL-mediated and T_{DTH}-mediated immune responses. These effector T cell-mediated immune responses may participate in the development of autoimmune diseases and in the rejection of grafts. Therefore,

it is suggested that the suppressive effect of MZR on clinical rejection and autoimmune disease is based on its suppression of cellular immunity, in addition to humoral immunity.

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