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ORIGINAL ARTICLE

Prophylactic treatment of antibody-mediated rejection with high-dose mizoribine and pharmacokinetic study*

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Summary

Although mizoribine (MZ), which inhibits inosine monophosphate dehydrogenase in the same way as mycophenolate mofetil, recently proved more effective when higher doses were administered than previously approved, neither the optimal dosage nor blood concentration has yet been clarified. We aimed at investigating the effect of high-doses of MZ on prevention of anti-donor antibody (Ab) production and acute Ab-mediated rejection (AMR) on the basis of the pharmacokinetic profile in a pig kidney transplantation model. Group 1 ($n = 5$) received cyclosporin microemulsion (6 mg/kg) and prednisolone (0.1 mg/kg). Groups 2, 3 and 4 (each $n = 5$) were treated, respectively, with 30, 10 and 3 mg/kg of MZ in addition to cyclosporin and prednisolone. The incidences of AMR in groups 1, 2, 3 and 4 were 5/5, 1/5, 3/5 and 5/5, respectively. Anti-donor IgG/IgM Ab levels (relative to pretransplantation levels) on day 14 in groups 1, 2, 3 and 4 were 10.3/9.3, 1.8/1.0, 2.3/1.8 and 6.5/3.5, respectively. While only 2 (28.6%) of seven pigs with $C_{max} > 3$ μg/ml during the first 2 weeks had AMR, 7 (87.5%) of eight pigs with $C_{max} < 3$ μg/ml elicited anti-donor Abs and experienced AMR ($P = 0.0406$). Effective C_{max} seemed to be over 3 μg/ml at minimum. Higher doses of MZ efficiently prevented AMR. However, therapeutic drug monitoring is essential before clinical application.

Introduction

To solve the current problem of organ donor shortage, transplantations involving a high risk of antibody-mediated rejection such as ABO blood group incompatibility or positive crossmatch have been actively attempted [1–3]. Consequently, the development of various additional treatments with antibody removal, splenectomy, anti-CD20 antibody and intravenous immunoglobulin (IVIG) has been improving graft survival. However, antimetabolite drugs including mycophenolate mofetil (MMF), azathioprine and cyclophosphamide basically play a pivotal role in B cell suppression. The inhibition of anti-donor antibody production would lead to a suppression of not only acute antibody-mediated rejection (AMR) but also chronic rejection. MMF, which has been recognized as an effective prophylactic treatment or rescue therapy for acute rejection, is now in use worldwide [4,5].

Mizoribine (MZ), which was originally developed in Japan and was authorized for marketing as an immunosuppressive agent for renal transplantation in 1984, was found to have a function similar to MMF as to its selective suppression of lymphocyte proliferation via the inhibition of inosine monophosphate dehydrogenese (IMPDH) [6,7]. MZ, which is a water-soluble imidazole nucleoside, is characterized by kidney excretion and a relatively short half-life (about 2 h) because of its low binding rate to plasma protein, in contrast with MMF, which shows bile excretion after being metabolized in liver and

approximately 18 h of half-life. Because its beneficial effect on organ transplantation has been demonstrated, MZ has been clinically used in China and Korea as well as Japan [8–10].

Recently, 2–3 mg/kg of MZ, which was originally registered as the clinically approved dosage, was found to be too small to obtain satisfactory efficacy, because such a dosage is much lower than the one for MMF (20–60 mg/kg) [8,11]. The efficacy of high-dose therapy (>5 mg/kg) has been shown in several studies. We previously reported that rescue therapy with high doses of MZ (30 mg/kg) could successfully reverse on-going AMR in a pig kidney allotransplantation model [7]. Although such a high dosage seems to be effective, neither the optimal dosage nor the blood concentration of MZ have yet been determined.

In this study, we investigated the effect of MZ on the prevention of acute AMR and analyzed the MZ pharmacokinetic (PK) in detail using the same pig kidney allotransplantation model as was previously reported to certainly cause AMR. Our purpose was to examine whether such a high-dose of MZ for prophylactic treatment would also prove both safe and effective, and to obtain the information on the optimum MZ blood level needed to sufficiently suppress acute AMR and anti-donor Ab production.

Methods

Animals

Pitman-Moore/Taiwan-Small Ear (NIBS miniature) pigs weighing 14–25 kg, and Landrace/Yorkshire pigs weighing 14–26 kg, were used as donors and recipients. All animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. Experimental protocols were approved by the Committee on Research Animal Care, Institute for Laboratory Animal Research, Nagoya University School of Medicine.

Immunosuppressive protocol, group classification and transplantation

Cyclosporin microemulsion (CsA), prednisolone (PSL), and MZ were administered through a gastrostomy tube as previously described. All recipient pigs were divided into the following four groups. Group 1 ($n = 5$) received CsA (6 mg/kg) and PSL (0.1 mg/kg) alone. Groups 2, 3 and 4 (each $n = 5$) were treated, respectively, with 30, 10 and 3 mg/kg of MZ in addition to CsA and PSL after transplantation.

The kidney of the Pitman-Moore/Taiwan-Small Ear (NIBS miniature) pig was heterotopically transplanted into the Landrace/Yorkshire pig [7]. Both native kidneys were removed immediately after transplantation. Biopsies of kidney grafts were conducted pre- and 1 h postreperfusion, and when the serum creatinine level was elevated above 3 mg/dl. The follow-up period was determined to be 3 weeks after transplantation, and all pigs were euthanized. Graft was resected for histopathological study at the end of the experiment (3 weeks after transplantation or at the time of death). The graft specimens were formalin-fixed and stained with hematoxylin–eosin (HE).

Diagnosis of acute rejection

Ab-mediated rejection was diagnosed according to the definitions given by the AMR criteria, an addition to the Banff '97 classification of renal allograft rejection [12]. C4d staining was not an option, as the cross-reactivity of commercially available polyclonal rabbit anti-C4d reagent with pig C4d could not be confirmed.

Blood test and pharmacokinetic study

Blood cell counts were measured once a week. Serum biochemical and electrolyte values were measured every day. A pharmacokinetic (PK) study of MZ was conducted on days 1, 4, 7 and 14. Blood levels of MZ (C0, C2, C4 and C6) were measured by HPLC [13].

Flow cytometry

The production of anti-donor antibodies (IgG, IgM) was analyzed by flow cytometry crossmatch using donor lymphocytes as previously described.

In brief, recipient pig serum was incubated with 2×10^5 donor T lymphocytes in phosphate-buffered saline (PBS) at 4 °C for 30 min. The T lymphocytes were then reacted with FITC-labeled F(ab)'2 fragment goat antiswine IgG (Jackson Immuno Research, West Grove, PA, USA) and goat anti-swine IgM (Bethyl Laboratories, Montgomery, TX, USA). The stained cells were subjected to flow cytometric analysis using EPIX XL (Beckman Coulter, Inc., Miami, FL, USA). The anti-donor IgG and IgM antibody levels were determined relative to the value at pretransplantation. Mean fluorescence intensity (MFI) was used for the calculations.

Statistical analysis

The Mann–Whitney U-test was used for statistical analysis of the values of anti-donor IgG/IgM antibodies. The relation between C_{max} and acute rejection was analyzed using Fisher's exact test. P-values < 0.05 were considered significant.

Results

All recipient pigs treated with CsA and PSL alone (group 1) developed acute rejection between days 5 and 9 (based on histological biopsy findings), and died due to renal failure several days after the onset of acute rejection (Table 1). Pathological examination revealed that all rejections were antibody-mediated [Banff grade, acute AMR (AAMR) type II–III], as described previously in this donor-recipient combination [7]. The incidences of AMR in MZ-treated groups 2 (30 mg/kg), 3 (10 mg/kg) and 4 (3 mg/kg) were 1/5, 3/5 and 5/5, respectively. All rejection episodes including biopsy-proven rejection without any notable increase in serum creatinine were observed between days 8 and 21. Rejection without creatinine increase on day 21 showed lower Banff grade (AAMR type I) than rejection with creatinine increase (AAMR type I-II, mainly II), but demonstrated evident feature of AMR.

In contrast to the control group (group1), all pigs in MZ-treated groups survived for 3 weeks and were then euthanized, except for one pig in group 2 with a functioning graft that suddenly died on day 19. Cause of death was unknown, as neither serious infection nor rejection was observed. MZ treatment seemed to reduce the progress of acute rejection. Blood count testing and biochemical analysis did not demonstrate adverse effects in any MZ-treated recipient pigs. Bone marrow suppression such as leukocytopenia was not observed.

Among MZ-treated groups (2, 3 and 4), the anti-donor IgG/IgM Ab levels on day 14 (2W), which were represented as relative values of pretransplantation levels were significantly higher in pigs with AMR ($n = 9$) than those in AMR-free pigs ($n = 6$; 4.9/3.0 versus 1.5/0.9, $P = 0.0184$/$P = 0.0032$; Fig. 1a,b). On day 21 (3W), anti-donor IgG/IgM levels did not increase and were maintained at the same levels as observed at pretransplantation in AMR-free pigs. In contrast, pigs with AMR showed a marked increase of anti-donor IgG/IgM levels (16.9/5.6).

Anti-donor IgG/IgM Ab levels on day 14 in groups 2, 3 and 4 were 1.8/1.0, 2.3/1.8 and 6.5/3.5, respectively

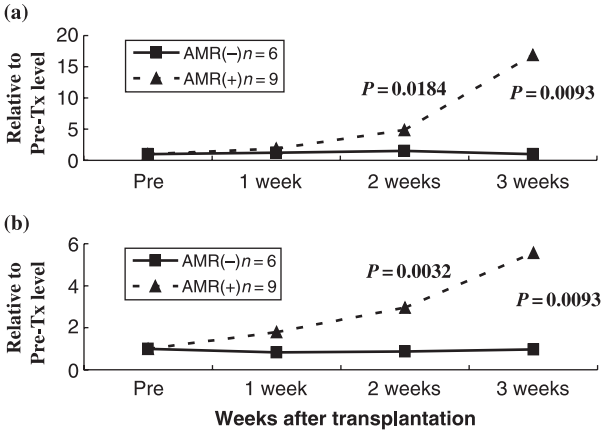

Figure 1 Acute rejection and changes in anti-donor antibodies after transplantation. Anti-donor IgG and IgM levels were represented in (a) and (b), respectively. Anti-donor antibodies were measured by flow cytometry using donor lymphocytes. Values were determined relative to pre-transplantation level. Anti-donor antibody levels in pigs with antibody-mediated rejection (AMR; closed triangle) were significantly higher than those in pigs without AMR (closed square) 2 and 3 weeks after transplantation.

(Fig. 2a,b). The values of anti-donor IgG and IgM in group 2 were significantly suppressed compared with those in group 4 ($P = 0.0472$, $P = 0.0090$).

Pharmacokinetic studies of MZ are shown in Fig. 3. Blood concentrations of MZ increased in a dose-dependent manner. On day 1, T_{max} seemed to be over 6 h, which was attributed to delayed gut absorption immediately after laparotomy. Stable absorption was observed after day 4, as peak serum concentration was estimated to occur between 2 and 4 h after administration.

The relation between C_{max} after transplantation and acute AMR was examined (Fig. 4). When C_{max} was maintained above 3 μg/ml during the first 2 weeks, AMR was observed in 2/7 (28.6%). In contrast, C_{max} of <3 μg/ml failed to suppress the production of anti-donor Abs, causing AMR in 7/8 (87.5%). That difference was statistically significant ($P = 0.0406$).

Table 1. Prevention of acute rejection by mizoribine (MZ) treatment.

Group (immunosuppression)	AR	Onset of AR (days)	AR-free survival (days)
1 (CsA + PSL)	5/5	5, 6, 7, 7, 9	
2 (CsA + PSL + MZ 30 mg/kg)	1/5	21*	>21, >21, >21, 19†
3 (CsA + PSL + MZ 10 mg/kg)	3/5	8, 11, 12	>21, >21
4 (CsA + PSL + MZ 3 mg/kg)	5/5	8, 11, 12, 21, 21*	

Most rejection episodes were diagnosed by histological findings as by well as the increase in serum creatinine.
*Biopsy-proven rejection without increase in serum creatinine.
†Sudden death with a functioning graft (cause of death unknown).

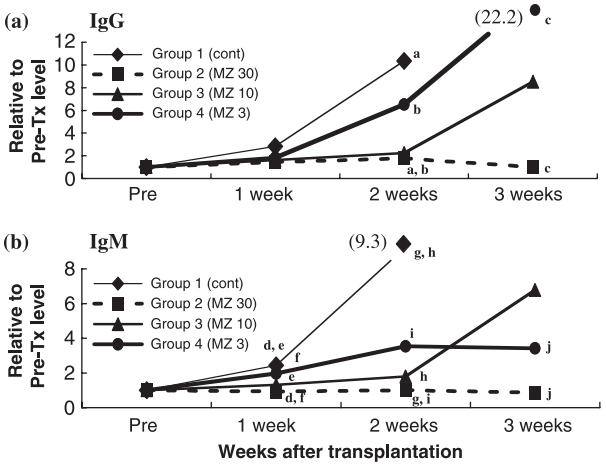

Figure 2 Changes in anti-donor antibodies by MZ dosage. Anti-donor IgG and IgM levels were represented in (a) and (b), respectively. Anti-donor antibodies were measured by flow cytometry using donor lymphocytes. Values were determined relative to pretransplantation level. Levels in group 1 (control), group 2 (30 mg/kg of MZ), group 3 (10 mg/kg of MZ) and group 4 (3 mg/kg of MZ) were depicted in closed diamond, square, triangle and circle, respectively. Anti-donor antibody levels in group 2 were maintained at a low level and were significantly lower than those in group 4. a: $P = 0.0283$, b: $P = 0.0472$, c: $P = 0.0143$, d: $P = 0.0090$, e: $P = 0.0472$, f: $P = 0.0283$, g: $P = 0.0090$, h: $P = 0.0472$, i: $P = 0.0090$, j: $P = 0.0275$.

Discussion

Mizoribine as well as MMF selectively inhibits IMPDH in the *de novo* pathway and suppresses purine synthesis, which indicates lymphocyte-specific immunosuppression, as lymphocytes are critically dependent on the *de novo* pathway for their proliferation. The efficient suppression of anti-donor Ab production was considered to be ascribed to the inhibition of both T- and B-cell proliferation.

Outside the transplantation field, MZ has proved clinically effective, safe and without serious adverse effects for treatment of nephrotic syndrome [14,15], IgA nephropathy [16,17], antineutrophil cytoplasm antibody (ANCA)-associated renal vasculitis [18,19], lupus nephritis [20,21] and rheumatoid arthritis [22,23].

Recently, higher doses of MZ ($\geqslant$5 mg/kg) compared with the clinically approved dosage (2–3 mg/kg) have been attempted for the treatment of renal diseases or autoimmune disease as well as organ transplantation, and demonstrated its versatile efficacy [8,11,24–26]. Very high doses of MZ (10 mg/kg) were effective in treating patients with frequently relapsing nephritic syndrome, showing that C2 levels reached over 3 μg/ml [24]. It was also reported that an effective peak MZ serum level over 2.5–3.0 μg/ml was necessary to control anti-ds DNA Ab production and to achieve satisfactory clinical efficacy for the treatment of lupus nephritis [25].

In the present study, using a pig kidney allotransplantation model, the maintenance of $C_{max} > 3$ μg/ml was proven to be effective in inhibiting the production of anti-donor Abs and the progress of AMR. This finding corresponds with the clinical results on nephrotic syndrome and lupus nephritis [24,25].

In clinical organ transplantation, it has just been reported that the therapeutic range would be 0.1–3 μg/ml, and that a trough level of >4–5 μg/ml would probably cause adverse effects [27]. Recent PK study in 40 kidney transplant recipients at a stable phase demonstrated that the average C_{max} level was 2.87 μg/ml [28]. However, conclusive results have not yet been reported.

Admittedly, one of the drawbacks in this study is that we could not fully investigate adverse effects, although neither bone marrow suppression nor liver dysfunction was observed in any pigs treated with such a high MZ dose. In particular, it should be noted that hyperuricaemia, which has been clinically identified as a major adverse effect, is not usually observed in pigs [7]. Furthermore, the results obtained in animal models, even when large animals were used, are not directly applicable to humans. As pigs seem to have a much lower bioavailability than humans (see company data on toxicokinetics), the dosage of 30 mg/kg in pigs would not be comparable with that in humans. However, the results obtained in our pig transplantation model (where AMR was certainly caused by anti-donor antibody production) would at least be considered to demonstrate the potential value of high-doses of MZ and to provide useful information on optimum blood levels for clinical trials.

Mizoribine has been reported to have a relatively lower incidence of the gastrointestinal disorders often observed in MMF-treated patients and of myelosuppression or liver dysfunction, which is readily caused by azathioprine or cylophosphamide [6,27].

Mizoribine was previously found to exhibit antiviral activities against influenza virus [29]. Moreover, a unique function recently reported showed that HCV RNA replication could be inhibited by MZ because of its similar structure to ribavirin [30]. Although much higher level ($IC_{50} = 26$ μg/ml) of MZ would be necessary for suppression of HCV RNA replication, clinically achievable concentration (1.3 μg/ml) of MZ was able to enhance the anti-HCV activity when used with IFN-alpha. This suggested that MZ might be a promising anti-HCV reagent in combination with IFN-alpha.

Thus, MZ, with its broad variety of functions, could, like MMF, potentially exert not only an inhibitory effect on anti-donor Ab production, but also produce additional benefits when a high-dose is applied. Its use would be expected to broaden the range of choices for the treatment or prevention of acute rejection, particularly of AMR.

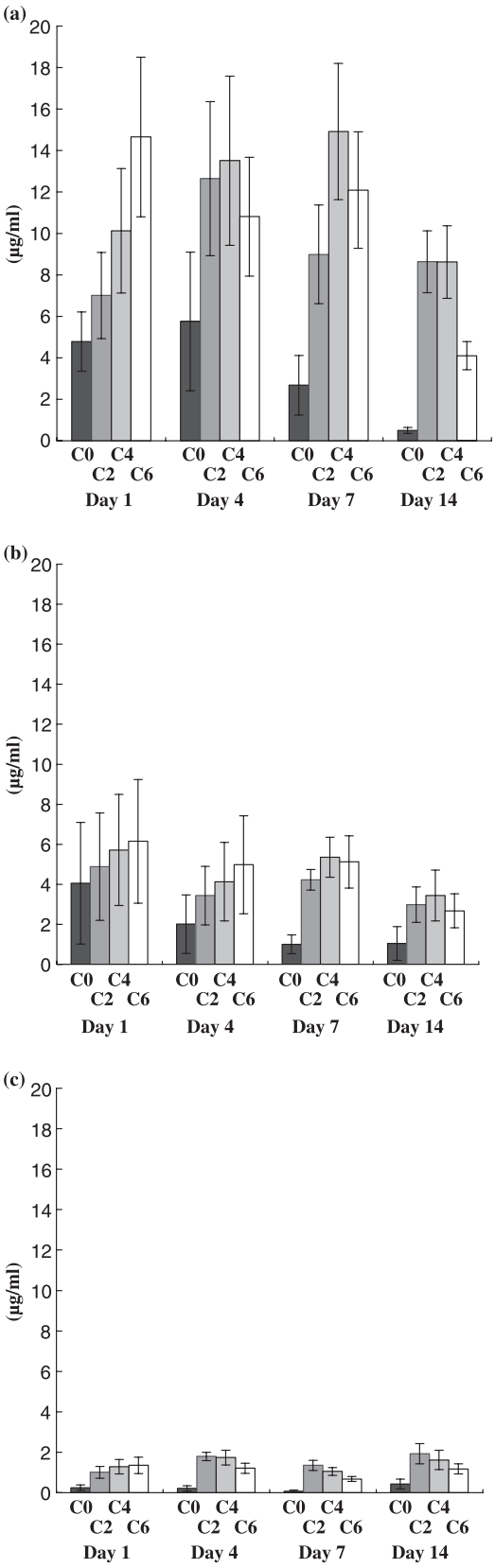

Figure 3 Pharmacokinetics by MZ dosage. (a) Group 2 (MZ 30 mg/kg), (b) group 3 (MZ 10 mg/kg), (c) group 4 (MZ 3 mg/kg). A pharmacokinetic study (C0, C2, C4 and C6) was conducted on days 1, 4, 7 and 14 after transplantation. Serum concentrations of MZ on days 1, 4, 7 and 14 were expressed as the mean ± SE. Serum levels of MZ were measured by HPLC and were dose-dependent. After day 4, stable absorption was observed, and T_{max} was estimated as between 2 and 4 h after transplantation.

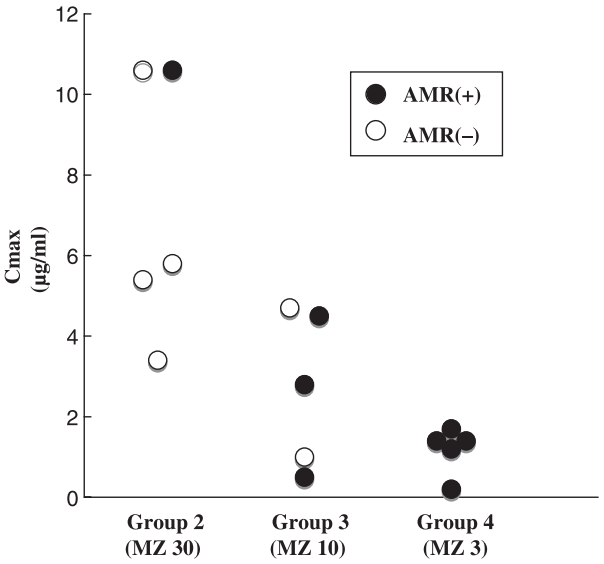

Figure 4 MZ blood concentration (C_{max}) and acute rejection. The lowest C_{max} levels during 14 days post-transplantation were plotted in groups 2, 3 and 4. Closed and open circles represent the levels with and without antibody-mediated rejection (AMR), respectively. When C_{max} was maintained at over 3 μg/ml during the first 2 weeks, AMR was observed in 2/7 (28.6%). In contrast, C_{max} < 3 μg/ml caused AMR in 7/8 (87.5%). That difference was statistically significant ($P = 0.0406$).

In conclusion, prophylactic treatment with high-dose MZ effectively prevented anti-donor Ab production and AMR in a pig kidney allotransplantation model. The effective C_{max} of MZ seemed to be over 3 μg/ml at minimum. Regarding MZ efficacy, higher doses than approved in the past were shown to be in no way inferior to MMF. However, the introduction of therapeutic drug monitoring would be essential not only to avoid adverse effects but also to ensure effective dosages.

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