Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Immunosuppressive efficacy of mycophenolate mofetil when compared with azathioprine and mizoribine against peripheral lymphocytes from renal transplant recipients

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Keywords
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), azathioprine, mizoribine, mycophenolate acid, mycophenolate mofetil, peripheral blood mononuclear cells.

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Received: 29 September 2004
Revised: 25 October 2004
Accepted: 15 December 2004

doi:10.1111/j.1432-2277.2005.00093.x

Summary

Mycophenolate mofetil is currently used instead of azathioprine in clinical transplantation. However, comparative studies for the immunosuppressive potency of anti-metabolites used for organ transplantation have not been well documented. We compared the pharmacological efficacy of mycophenolic acid (MPA), 6-meraputopurine (6-MP), and mizoribine (MZ) for inhibiting purine synthesis of peripheral blood mononuclear cells (PBMCs) *in vitro* by a mitogen assay procedure. PBMCs were obtained from 18 renal transplant recipients before operation and 18 healthy subjects. The inhibitory efficacy of 6-MP against concanavalin A-induced PBMC blastogenesis exhibited large variations between subjects in both recipients and healthy subjects. In contrast, the pharmacological efficacy of MPA on PBMC blastogenesis showed the smallest inter-individual variation of all the purine synthesis inhibitors examined. Furthermore, the effects of MPA were almost similar in the recipients and healthy subjects. The pharmacological efficacy of MZ against PBMC blastogenesis was weaker than that of the other two agents and the inter-individual variation of MZ IC_{50} against PBMCs of the patients was larger than that of MZ IC_{50} against PBMCs of healthy subjects. Reproducible immunosuppressive efficacy of MPA compared with other purinesynthesis inhibitors could be expected from the viewpoint of MPA pharmacodynamics against PBMCs in renal transplantation.

Introduction

Purine synthesis inhibitors have been used as important agents for prevention of allograft rejection in organ transplantation. They continue to be used in combination with glucocorticoid and calcineurin inhibitors. In organ transplantation, three purine synthesis inhibitors, mycophenolate mofetil (MMF), azathioprine (Az) and mizoribine (MZ) are generally used for treatment. MMF is a relatively new immunosuppressive agent belonging to a class of purine synthesis inhibitors that has shown promise mainly in renal transplantation. A lower rate of treatment failure resulting from biopsy-proven allograft rejection has been reported in renal transplantation with MMF in comparison with Az [1]. Furthermore, in case of first use of anti-lymphocyte therapy at rejection episode, the incidence of treatment failure in MMF-treated patients was reported to be lower than that in Az-treated patients [2].

Accordingly, MMF is currently used, instead of Az, for prevention of rejection episode in transplantation. MMF selectively inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH) in the *de-novo* pathway of purine synthesis [3]. MZ monophosphate is the active metabolite of the immunosuppressive agent MZ, and the

metabolite is a potent inhibitor of IMPDH [4]. Az is a pro-drug of 6-mercaptopurine (6-MP). Az is cleaved to 6-MP, which in turn can be converted to 6-MP nucleotides leading to inhibition of *de-novo* purine synthesis or anabolism to thio-IMP, which, as a pseudo-nucleotide, can interfere with the salvage pathway of purine sysnthesis [3].

In our previous study, we compared the immunosuppressive pharmacological efficacy of prednisolone and prednisolone sodium succinate *in vitro* using human peripheral blood mononuclear cells (PBMCs) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [5]. We found that the immunosuppressive potency of prednisolone sodium succinate was several fold lower than that of prednisolone *in vitro* [5]. Prednisolone has low solubility in water, and therefore it must be esterified to make a water-soluble injection formulation. Similarly, MMF is a pro-drug of mycophenolate acid (MPA), and the bioavailability of MPA was improved by esterification to be MMF [6]. However, the pharmacological efficacies of Az and its pro-drug 6-MP against blastogenesis of human PBMCs were almost equal. AZ is effective after conversion to 6-MP in PBMCs *in vitro* [7]. The suppressive potency of immunosuppressive drugs against *in vitro* blastogenesis of PBMCs from patients can predict the clinical immunosuppressive efficacy of the drugs in renal transplant recipients [8].

In the present study, we compared the pharmacological efficacy of MPA, 6-MP and MZ for inhibiting mitogen-induced proliferation of immune cells *in vitro* using PBMCs from transplant recipients before operation and from healthy subjects. We also examined the relationship between the PBMC suppressive efficacy of MPA and the clinical outcome of the recipients treated with MMF.

Materials and methods

Reagents

Mycophenolate acid and 6-MP were obtained from Wako Chemical Co. (Osaka, Japan), MZ was provided by Asahikasei Co. (Tokyo, Japan). MTT was from Sigma Chemical Co. (St Louis, MO, USA). Ficoll-Paque was obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). RPMI 1640 medium, fetal bovine serum, and Hank's balanced salt solution were obtained from Gibco Laboratories (Rockville, NY, USA). Concanavalin A was from Seikagaku Kogyo Co. (Tokyo, Japan). All other reagents were of the highest grade available.

Subjects

After informed consent was obtained, heparinized venous blood (20 ml) was taken from 18 transplant recipients (12 males and 6 females) before operation. The mean (SD) age of these recipients was 35.6 (13.6) years. Blood sampling was also carried out in 18 healthy subjects aged 29.1 (6.6) years (14 males and 4 females). All transplant recipients received a primary renal allograft from living donors after blood sampling for analysis of their PBMC response to immunosuppressive agents *in vitro*. There was no significant difference in mean age and male/female ratio between the patients and healthy subjects. All 18 patients underwent renal transplantation from July 2002 to October 2003 at Niigata University Medical and Dental Hospital. The study was approved by the Ethics Review Board of the Medical Faculty of Niigata University.

These patients were treated with maintenance immunosuppressive therapy after transplantation, which consisted of a combination of methylprednisolone, basiliximab, either cyclosporin A (Neoral) or tacrolimus prograf cap; (Fujisawa Co., Osaka, Japan), and MMF celcept (250 mg) cap; (Chungai Co., Tokyo, Japan). Thus, all of these patients received oral MMF therapy. The starting doses of these agents were 125 mg/day for methylprednisolone, 2–3 mg/kg/day for cyclosporine intravenously (or 8 mg/kg/day orally), 0.05 mg/kg/day for tacrolimus intravenously (or 0.2 mg/kg/day orally), and 1000 mg b.i.d. for MMF. The healthy subjects had no history of taking immunosuppressive agents, including purine synthesis inhibitors. To measure the response of PBMCs to the drugs *in vitro*, a drug sensitivity test [8,9] was carried out in each patient before transplantation or in each healthy subject as described below.

Isolation of PBMCs

Venous blood was taken and heparinized at about 4:00 PM just before immunosuppressive agents were administered for renal transplantation on a day on which hemodialysis was not performed. Isolation and culture of PBMCs were carried out according to the method described previously [8,9]. In brief, 5 ml of heparinized blood was loaded onto 4 ml of Ficoll-Paque and centrifuged at $900 \times g$ for 20 min at room temperature. The buffy coat was taken and rinsed three times with Hank's balanced salt solution. PBMCs, including lymphocytes, were suspended in RPMI 1640 medium containing 10% fetal bovine serum to a cell density of 1×10^6 cells/ml.

PBMC culture and evaluation of drug potency

The cell suspension prepared as described above was placed into each well of microplates with 96 flat-bottomed wells. Saline containing concanavalin A was added to each well to a final mitogen concentration of 5.0 μg/ml. Subsequently, diluted sodium hydroxide solution containing 6-MP or ethanol solution containing MPA was added

to give a final drug concentration of 0.01, 0.1, 1, 10, 100, 1000, 10 000 or 100 000 nM. Aqueous solution containing MZ was added to give a final drug concentration of 1, 10, 100, 1000, 10 000, 100 000, 1 000 000, or 10 000 000 nM. The same volume of each vehicle solution was added to control wells. The plates were incubated for 4 days in an atmosphere of 5% CO_2 at 37 °C.

MTT assay

After 4 days of culture, 10 μl of 5 mg/ml MTT solution dissolved in saline was added to each well and then the cultures were re-incubated under 5% CO_2 at 37 °C for 4–5 h [5,7,10]. The plates were centrifuged at 375 × g for 5 min to precipitate cells and formazan produced by growing cells. Aliquots of the supernatant were removed from each well and dimethylsulfoxide was added followed by shaking of the plate on a microshaker for 10 min to dissolve the formazan crystals. The absorbance was read with a microplate reader at 550 nm. Dose–response curves were plotted, and the concentrations of drugs that gave 50% inhibition of cell growth (IC_{50}) were calculated.

Statistical analysis

Because of their skewed distribution, IC_{50} raw data were log-transformed before performing statistical analysis. Continuous variables were compared using Student's *t*-test. When necessary, the Welch's *t*-test was used for unequal variances. Frequency analysis was performed with Fisher's exact test. The relationship between continuous variables was investigated by means of Pearson's correlation coefficient (*r*). The homogeneity of variances of IC_{50} values among three groups (6-MP, MPA, MZ) was assessed by the Bartlett test. The variance of IC_50 between any two groups (6-MP MPA, MPA MZ, and 6-MP MZ) was assessed using the *F*-test. For multiple group comparisons, the significance of individual differences was evaluated by using the Scheffe test. Values of $P < 0.05$ were considered to indicate statistical significance. Statview 5.0 statistical software (SAS Institute, Cary, NC, USA) was used for statistical analysis.

Results

The effects of 6-MP, MPA and MZ on the *in vitro* blastogenesis of PBMCs from renal transplant recipients before operation and from healthy subjects were compared. Typical dose–response curves for 6-MP, MPA and MZ on the mitogen-induced blastogenesis of PBMCs obtained from one recipient are shown in Fig. 1. All purine synthesis inhibitors suppressed blastogenesis dose dependently.

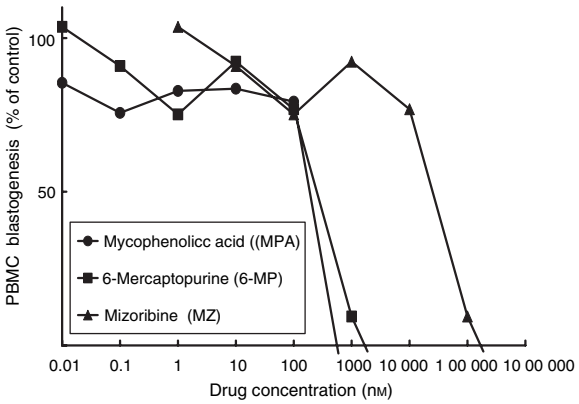

Figure 1 Typical dose–response curves of the purine synthesis inhibitors on Con A-stimulated blastogenesis of PBMCs from a transplant recipient before operation.

In PBMCs from the recipients, the mean (SD) of 6-MP IC_{50} values was 6041.4 (22800.1) nM ($n = 18$). The range showed a huge variation from 0.01 to 100 000 nM. Similarly, in healthy subjects, the mean (SD) of 6-MP IC_{50} values was 429.5 (879.4) nM, and the range showed a large deviation from 0.01 to 3828.9 nM. In both patients and healthy subjects, the individual differences of the pharmacological efficacy of 6-MP (range of 6-MP IC_{50}s) against PBMC blastogenesis were the largest for the purine synthesis inhibitors presently examined (Figs 2 and 3).

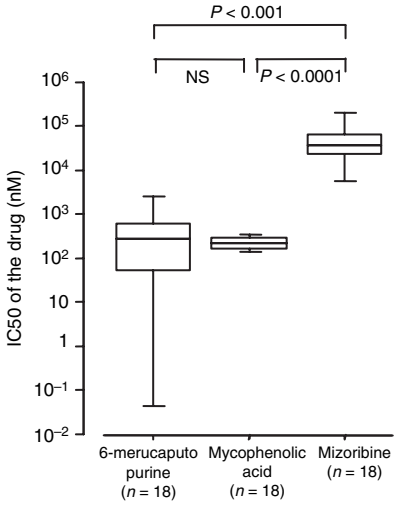

Figure 2 IC_{50} values of purine synthesis inhibitors against mitogen-induced blastogenesis of PBMCs from renal transplant recipients. Median IC_{50} values (282.9, 222.1 and 36292.4 nm for 6-MP, MPA and MZ, respectively) are shown by horizontal bars in the boxes. The horizontal boundaries of the boxes represent the first and third quartiles. The vertical bars indicate the range from the 10th to the 90th percentile. Scheffe's test for multiple comparisons was applied.

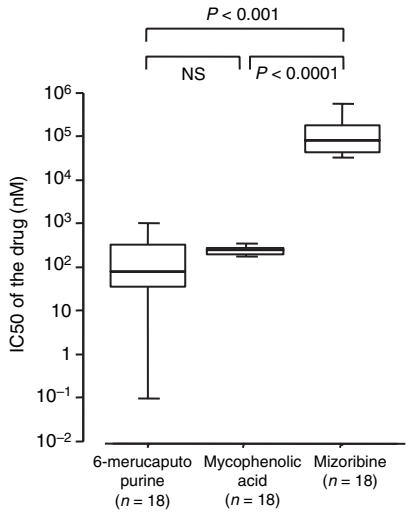

Figure 3 IC_{50} values of purine synthesis inhibitors against mitogen-induced blastogenesis of PBMCs from healthy subjects. Median IC_{50} values (82.3, 248.9 and 84649.0 nm for 6-MP, MPA and MZ, respectively) are shown by horizontal bars in the boxes. The horizontal boundaries of the boxes represent the first and third quartiles. The vertical bars indicate the range from the 10th to the 90th percentile. Scheffe's test for multiple comparisons was applied.

The mean (SD) of MPA IC_{50} values against the blastogenesis of patients PBMCs was 234.0 (74.2) nM. The IC_{50} range, in contrast to the range of Az, showed more limited deviation from 116.7 to 372.4 nM. Similarly, in healthy PBMCs, the mean (SD) of MPA IC_{50} values was 252.8 (71.5) nM, with limited deviation from 156.1 to 458.7 nM. The IC_{50} range, in contrast to the range of Az, showed limited deviation (Figs 2 and 3).

The mean (SD) of MZ IC_{50} values against patient's PBMCs was 65687.1 (66162.3) nM. The range of IC_{50} showed relatively large deviations from 3347.4 to 214433 nM. The median of MZ IC_{50} was 36292.4 nM. The mean (SD) of MZ IC_{50} values against healthy PBMCs was 181029.7 (240889.9) nM, and the range was relatively small, from 29987.3 to 913280.2 nM, compared with the range of IC_{50}s against PBMCs of the patients. The potency of MZ was weakest among the purine synthesis inhibitors examined. The variation of individual pharmacological efficacies of MZ in healthy subjects (range of MZ IC_{50}) was small, while the inter-individual variation of MZ effects on patient's PBMCs (range of MZ IC_{50}) was relatively large (Figs 2 and 3).

Multiple group comparisons performed using the Scheffe test revealed the significance of individual differences, as shown in Figs 2 and 3. The values of log-transformed IC_{50} of MZ were significantly lower in patients than in healthy subjects (Table 1, $P = 0.0149$). However, for 6-MP IC_{50}s and MPA IC_{50}s, there was no significant difference between patients and healthy subjects.

Table 1. Characteristics of subjects and comparisons of IC_{50} value between candidates for kidney transplantation and healthy subjects.

	Patients ($n = 18$)	Healthy subjects ($n = 18$)	*P*-value
Male (%)	12 (66.7)	14 (77.8)	NS
Female (%)	6 (33.3)	4 (22.2)	NS
Mean age (SD)	35.6 (13.6)	29.1 (6.6)	NS
Mean HLA match number (SD)	2.78 (4.7)	–	
IC_{50} (nm)			
6-mercaputopurine	6041.4 (22800.1)	429.5 (879.4)	–
MPA	234.0 (74.2)	252.8 (71.5)	–
MZ	65687.1 (66162.3)	181029.7 (240889.9)	–
Log-transformed IC_{50} (nm)			
6-mercaputopurine	1.959 (1.793)	1.740 (1.483)	NS
MPA	2.346 (0.147)	2.387 (0.118)	NS
MZ	4.587 (0.507)	4.998 (0.452)	0.0149

Data are mean (SD) unless otherwise stated. IC_{50}, 50% inhibition of cell growth; NS, Not significantly different between each group.

Finally, we examined the relationship between PBMC sensitivity and MPA *in vitro* before the operation and the clinical outcome in 18 renal transplant recipients. All recipients received oral MMF therapy. The number (%) of rejection episodes was five (27.8%) during the 6 months after transplantation. The number of patients (%) who were primarily treated with cyclosporine was 14/18 (77.8%), while the number of recipients primarily treated with tacrolimus was 4/18 (22.2%). The incidence of rejection episode in cyclosporine-treated recipients was 4/14 (28.6%), while the incidence in tacrolimus-treated recipients was 1/4 (25.0%) 6 months after transplantation. Conversion of calcineurin inhibitors from cyclosporin A to tacrolimus was carried out in 2/14 (14.2%). Conversion of calcineurin inhibitors from tacrolimus to cyclosporin A was not carried out. The number of adverse events which were considered to arise from immunosuppressive drugs was three (17.6%). The incidence of adverse gastrointestinal events was one (5.6%) and that of medicamentosus hepatopathy was two (11.1%). The two patients who experienced adverse effects were changed from MMF to other purine synthesis inhibitors. The incidence of adenovirus infection was two (11.8%), and incidence of CMV infection four (22.2%) (Table 2). The number of ABO-incompatible donors was one (5.6%). Graft survival at 6 months was achieved in 17 cases (94.4%). Patient survival at 6 months after renal transplantation was 18 (100%).

Blood monitoring of cyclosporin A at rejection episode($n = 4$) was carried out, and the cyclosporin A trough level (SD) was 258 (81.2) ng/ml. The data for Neoral C2 monitoring revealed 1091.5 (334.6) ng/ml.

Table 2. Clinical events in renal transplant recipients within 6 month after transplantation.

Base of CNI	No. of acute rejection (%)	No. of CNI conversion (%)
Cyclosporin A ($n = 14$)	4/14 (28.6)	2/14 (14.2)
Tacrolimus ($n = 4$)	1/4 (25.0)	0/4 (0)

Adverse event	Transplant recipient ($n = 18$), (%)
Adverse event from immunosuppressive agent	3 (17.6)
Gastrointestinal adverse event	1 (5.6)
Medicamentosus hepatopathy	2 (11.1)
Patients converted from MMF to another purine synthesis inhibitor	2 (11.1)
Adenovirus infection	2 (11.8
CMV infection	4 (22.2)

CNI, Calcineurin inhibitor.

Table 3. Monitoring of calcineurin inhibitor blood concentration at rejection episode.

Cyclosporin A ($n = 4$)	
Co	258 ng/ml (81.2)
C2	1091.5 ng/ml (334.6)
AUC_{0-4}	3187 ng h/ml (841.2)
Tacrolimus ($n = 1$)	
Co	18.8 ng/ml (0)
Rejection episode time (day)	24.5 (11.7)

Data are mean (SD).

AUC_{0-4} was 3187 (841.2) μg h/l. Similarly, blood monitoring for trough level of tacrolimus revealed its concentration as 18.8 ng/ml at the acute rejection episode ($n = 1$) (Table 3).

Discussion

In this study, we compared the anti-proliferative efficacy of 6-MP, MPA and MZ *in vitro* against mitogen-stimulated PBMCs of renal transplant recipients before the operation and healthy subjects. The individual variation of the effect of MPA in both patient's PBMCs and healthy PBMCs was smallest among the purine synthesis inhibitors examined. Furthermore, the potency of MPA to suppress PBMC blastogenesis was almost equal in transplant recipients and healthy subjects. In healthy PBMCs, the individual variation of the pharmacological efficacy of MZ was also small. However, the potency of MZ to suppress PBMC blastogenesis was much less than that of MPA or AZ. Furthermore, the variation of MZ IC_{50}s in PBMCs of renal transplant recipients was larger than that in healthy subjects. Thus, the efficacy of MPA for suppressing blastogenesis of PBMCs from renal transplant recipients was similar to that of 6-MP, and was much superior to that of MZ. In addition, the effect of MPA against PBMC blastogenesis exhibited the minimum individual variation among the transplant recipients.

In the present study we examined the clinical outcome and pharmacological efficacy of the drugs. The clinical outcome such as adverse effect of MPA or acute rejection episodes did not correlate with the *in vitro* pharmacological efficacy of MPA. Therefore, the clinical outcome of the recipients in these recipients appeared to be influenced by other factors, including sensitivity of PBMC to glucocorticoid and/or cyclosporin A/tacrolimus.

Previously we demonstrated that in PBMCs of chronic renal failure (CRF) patients, the variation of the pharmacological efficacy of prednisolone is larger than that in PBMCs of healthy subjects. In contrast, the effect of methylprednisolone against PBMC blastogenesis is almost the same in CRF patients and healthy subjects [8]. Furthermore, the individual variation of the suppressive effect of methylprednisolone against PBMC blastogenesis was smallest among the four glucocorticoids examined. Based on these observations, we previously suggested that the most appropriate glucocorticoid to achieve constant immunosuppressive efficacy in renal transplantation is methylprednisolone [8]. From the data obtained in the present study, we suggest that a similar pattern occurs for purine synthesis inhibitors. As described above, the variation of the individual pharmacological efficacy of MPA against PBMC blastogenesis was smallest among the purine synthesis inhibitors, and therefore MMF may be the most effective purine synthesis inhibitor in renal transplantation. Some clinical data concerning the use of purine synthesis inhibitors in the transplant recipients support this idea. Recipients treated with MMF to exhibit a significantly low incidence of late-treatment failure of allograft and biopsy-proven rejection when compared with recipients treated with Az in renal transplantation [1]. Furthermore, in case of first use of anti-lymphocyte therapy at rejection episode, the incidence of treatment failure in MMF-treated patients was lower than that in Az-treated patients [2]. MMF is also superior to AZ for prolonging graft survival in heart transplantation [11], liver transplantation [12], and lung transplantation [13,14].

There are two major pathways of purine synthesis, and cell types and tissues can be classified according to their dependence on the *de-novo* and salvage pathways of purine synthesis. Most tissues, including brain tissues, are able to use both of these pathways, and are thus in an intermediate category. However, lymphocytes show extreme dependence on the *de-novo* synthesis. MMF is a potent, noncompetitive, reversible inhibitor of the enzyme

IMPDH, and therefore, this drug selectively suppresses the proliferation of both T and B lymphocytes [15]. Similarly, MZ is converted to MZ monophosphate as an active metabolite, and this metabolite exhibits potent IMPDH inhibitory activity in *de-novo* pathways of purine synthesis [4]. Therefore, both MMF and MZ can selectively block the *de-novo* pathways of purine synthesis in proliferating lymphocytes. In the present study, the individual variation of IC_{50}s of both MPA and MZ were relatively small compared with of AZ, suggesting that the individual variation of the anti-lymphoproliferative efficacy of these IMPDH-inhibitors among patients should be minimal. In contrast, AZ and its active metabolite, 6-MP, block several enzymes involved in purine synthesis through comparative inhibition [3], which may result in individual variation of the anti-PBMC effect of AZ, as observed in the present study (Figs 2 and 3).

In summary, individual variation of the anti-PBMC effect of MPA was smallest among the purine synthesis inhibitors tested. Furthermore, the pharmacological efficacy of MPA was superior to the efficacy of MZ and was almost equal to that of AZ. Many researchers have found a close correlation between PBMC suppression by immunosuppressive drugs *in vitro* and clinical efficacy of the drugs in renal transplantation, and thus the present data support the notion that MMF could be used as a first-choice purine synthesis inhibitor with little individual variation of its efficacy in renal transplantation.

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