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ORIGINAL ARTICLE

Mycophenolate mofetil inhibits T-cell proliferation in kidney transplant recipients without lowering intracellular dGTP and GTP

Sanjay U. C. Sankatsing,[1] Jan M. Prins,[1] Si-La L. Yong,[2] Jeroen Roelofsen,[3] André B. P. van Kuilenburg,[3] Steve Kewn,[4] David J. Back,[4] Frederike J. Bemelman[2] and Ineke J. M. ten Berge[2]

1 Division of Infectious Diseases, Tropical Medicine and AIDS, Department of Internal Medicine, and Center for Infection and Immunity Amsterdam (CINIMA), University of Amsterdam, Amsterdam, The Netherlands
2 Renal Transplant Unit, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
3 Department of Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
4 Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool, UK

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Correspondence
Sanjay U. C. Sankatsing, Division of Infectious Diseases, Tropical Medicine and AIDS, Department of Internal Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel.: +31 20 5664380; fax: +31 20 6972286; e-mail: s.u.sankatsing@amc.uva.nl

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Summary

To study if mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), indeed inhibits T-cell proliferation in kidney transplant recipients by lowering intracellular deoxyguanosine triphosphate (dGTP) and guanosine triphosphate (GTP) levels. Blood was drawn from 11 kidney transplant recipients. *Ex vivo* T-cell proliferation was measured by stimulation with phytohemagglutin (PHA) and anti-CD3 monoclonal antibody (mAb). Plasma MPA levels and intracellular dGTP and GTP in peripheral blood mononuclear cells were measured. MMF induces a significant decrease in T-lymphocyte proliferation at all time points (i.e. 24 h, 10 days and 8 weeks) after stimulation with both PHA ($P = 0.001$, 0.002 and 0.013 respectively) and anti-CD3 mAb ($P = 0.004$, 0.004 and 0.005 respectively). There was no significant change in intracellular dGTP ($P = 0.31$, 0.16 and 0.35) or GTP levels ($P = 0.99$, 0.32 and 0.49) between baseline and day 1, day 10 or week 8. All MPA levels were above the minimal required concentration for the inhibition of lymphocyte proliferation. MMF inhibits T-lymphocyte proliferation in kidney transplant recipients without lowering intracellular dGTP or GTP levels. This suggests another mechanism underlying its immunosuppressive capacity.

Introduction

Mycophenolate mofetil (MMF) is used to prevent rejection after solid organ transplantation and is nowadays an important drug for kidney transplant patients [1,2]. It is a prodrug that is rapidly converted to its active metabolite mycophenolic acid (MPA), which selectively inhibits inosine 5′-monophosphate dehydrogenase (IMPDH), a key enzyme in the purine *de novo* synthetic pathway of guanosinenucleotides [3,4]. There are two pathways for guanosine production, the *de novo* pathway and the salvage pathway. In activated lymphocytes, the *de novo* pathway is dominant [3]. Blocking the conversion of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) by inhibiting IMPDH depletes guanosine triphosphate (GTP) and deoxyguanosine triphosphate (dGTP) pools. This depletion of dGTP pools is thought to be responsible for diminished proliferation of T lymphocytes *in vitro* and is thought also to underlie – at least in part – the efficacy of MMF *in vivo* [3].

Previously, we performed a randomized trial in which HIV-1-infected patients were treated either with highly active antiretroviral therapy (HAART) in combination with MMF or with HAART alone. The rationale of this study was that by inhibiting lymphocyte proliferation, the availability of target cells for HIV infection would be lowered. Furthermore, because of the supposed effect of MPA on the dGTP pools, the depletion of intracellular

dGTP could have a direct antiretroviral effect and could enhance the antiretroviral effect of the guanosine nucleoside analogue abacavir [5]. Although polyclonally induced lymphocyte proliferation as detected by ^{3}H-thymidine incorporation was significantly lower in patients treated with MMF, no difference was observed in the dGTP pools in peripheral blood mononuclear cells (PBMCs) between patients who were treated with MMF and those who were not. There were also no differences in the increase of CD4 T-cell counts or decrease in plasma HIV RNA between the two patient groups [5–7]. To explain these findings, we hypothesized that the cell develops a compensation mechanism, for instance by using the salvage pathway for guanosine nucleotide production, thereby masking decreased dGTP levels.

To explore this further, we studied renal transplant recipients just before and at different time intervals after the start of immunosuppressive drug therapy including MMF. We measured the intracellular dGTP and GTP pools in parallel to T-cell activation markers and proliferative capacity.

Methods

Patients

Kidney transplant patients receiving a kidney from a living donor in our hospital were eligible for this study if they were above 18 years of age and never used MMF before. Apart from MMF in a dosage of 1000 mg twice daily orally, they received the CD25 monoclonal antibody basiliximab, 20 mg i.v. at day 0 and 20 mg i.v. at day 4 postoperatively, as well as prednisolone 10 mg daily and cyclosporine aiming at trough levels of 100 ng/ml. Written informed consent to obtain blood samples for the purpose of this study was obtained from all patients.

Pharmacokinetics

The plasma MPA trough concentration was measured 24 h after the first dosage (i.e. 12 h after the last dosage and just before the third dosage) and after 10 days and 8 weeks of treatment. For each measurement, 7 ml heparinized blood was obtained. Plasma concentrations of MPA were measured with a liquid chromatographic assay, as described earlier, which has been validated in the ranges of 0.01–10 mg/l for 200 μl plasma samples [8].

Lymphocyte proliferation assays

At baseline, before the patient received MMF or other immunosuppressive drugs, after 24 h, 10 days and 8 weeks blood was collected and whole-blood lymphocyte cultures were performed as described previously [9]. In summary, heparinized blood from patients was 1:10 diluted with Iscove's Modified Dulbecco's Medium, supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) and β-mercapto-ethanol (1 μg/ml). Aliquots of 150 μl were incubated with phytohemagglutin (PHA; Welcome, Beckenham, UK), final concentration 5 μg/ml or with coated anti-CD3 T3/4.E mAb (Sanquin, Amsterdam, the Netherlands) 1/1000. All incubations were performed in U-bottomed wells of microtitre plates (Costar, Corning, NY, USA) at 37 °C in a humidified atmosphere of 5% CO_2 for 96 h. The cells were harvested and proliferation was determined by measurement of incorporation of 3H-thymidine (0.2 μCi/well, specific activity 2 Ci/mmol), added 4 h before the end of incubations, using liquid scintillation counting. All incubations were performed in triplicate and the median values of counts per minute were used in further analyses.

Intracellular GTP

At baseline before start of MMF, and after 24 h, 10 days and 8 weeks heparinized blood was collected and lymphocytes were isolated by Ficoll–Hypaque density centrifugation (Pharmacia Biotech AB, Uppsala, Sweden). Ribonucleotides were extracted by adding 150 μl of ice-cold 0.4 M perchloric acid to a cell pellet of $5–10 \times 10^6$ cells. After 10 min on ice, the suspension was centrifuged at 11 000 *g* at 4 °C for 15 min. The supernatant was removed and neutralized with approximately 7.5 μl 5.0 M K_2CO_3. After centrifugation (11 000 *g* at 4 °C for 5 min), the supernatant was saved and used for HPLC analysis. The pellet containing total protein was dissolved in 500 μl 0.2 M NaOH and the protein content was measured with a copper-reduction method using bicinchonic acid, essentially as described by Smith *et al.* [10].

Nucleotide profiles were determined by ion exchange HPLC using a Whatman Partisphere SAX column (4.6 × 125 mm, 5 μm particle size) and a Whatman AX (10 × 25 mm) guard column. Buffer A consisted of 9 mM $NH_4H_2PO_4$ (pH 3.5) and buffer B consisted of 325 mM $NH_4H_2PO_4$ (pH 4.4) and 500 mM KCl. Nucleotides were eluted with a gradient from 100% buffer A to 90% buffer B in 60 min at a flow-rate of 1 ml/min.

Intracellular dGTP

At the same time points as mentioned above, a separate 32 ml blood sample was obtained in cell preparation tubes with sodium citrate and PBMCs were isolated, for the measurement of dGTP with an enzymatic assay as described earlier [7,11].

Statistical analysis

Data were analysed with SPSS 11.5.2 (SPSS Inc., Chicago, IL, USA). Median and interquartile range (IQR) of MPA plasma levels, thymidine incorporation, and dGTP and GTP levels were calculated. The *T*-test was used to analyse the changes in thymidine incorporation and in intracellular nucleotide levels.

Results

Patients

Between May 2003 and April 2004, 11 patients were included (nine males and two females) with a median age of 47 years (IQR 31–53 years), who each received a first kidney transplant. In the past, none of them had been treated with immunosuppressive drugs.

Pharmacokinetics of MPA

All MPA plasma trough levels (26 samples, Fig. 1) were above the IC50 of 0.14 μg/ml, above which a decrease in lymphoproliferative capacity as measured by thymidine incorporation ([^{3}H]-TdR incorporation) may be expected [12]. The median MPA plasma level for all samples was 2.00 μg/ml (IQR 0.55–2.84 μg/ml).

Immunology

After initiation of MMF treatment, in all patients, a significant decrease in proliferative capacity of lymphocytes as measured by [^{3}H]-TdR incorporation was observed after whole-blood stimulation with both PHA (Fig. 2a) and aCD3 mAb (Fig. 2b). This decrease in proliferation as compared to baseline was observed not only after 24 h ($P = 0.001$ for PHA and $P = 0.004$ for aCD3 mAb stimulation) but also after 10 days ($P = 0.002$ for PHA and $P = 0.004$ for aCD3 mAb stimulation) and after 8 weeks ($P = 0.013$ for PHA and $P = 0.005$ for aCD3 mAb stimulation).

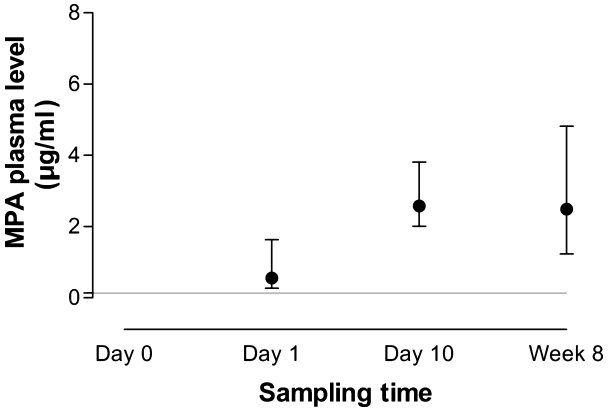

Figure 1 Median (IQR) MPA plasma trough levels. The *x*-axis shows the time in days after the first MMF dosage. The horizontal dotted line represents the minimum required MPA plasma level (0.14 μg/ml).

Intracellular dGTP and GTP

There was no significant change in intracellular dGTP levels between baseline and day 1, 10 or week 8 ($P = 0.31$, 0.16 and 0.35; Table 1 and Fig. 3a). On the contrary,

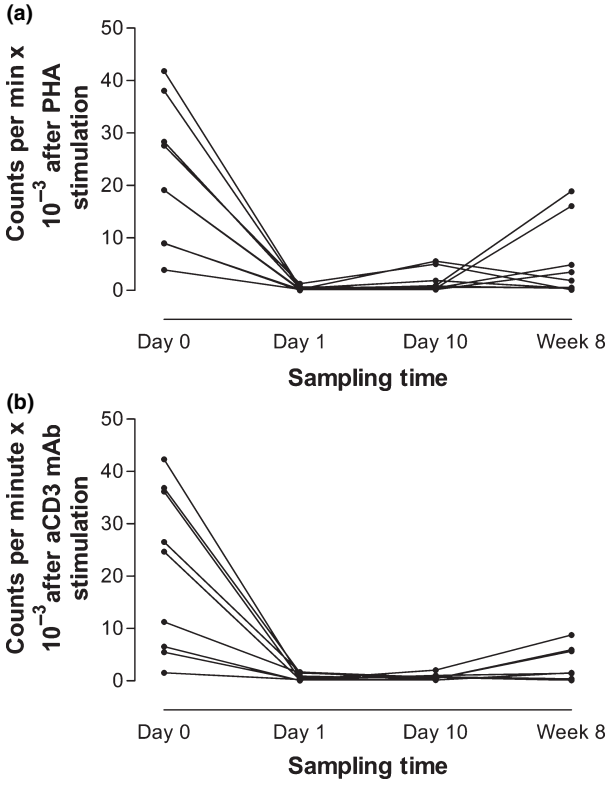

Figure 2 Proliferative capacity of lymphocytes measured with [^{3}H]-TdR incorporation in counts per minute of each patient (a) after PHA and (b) after aCD3 mAb stimulation. The *x*-axis shows the time in days after the first MMF dosage.

Table 1. Intracellular nucleotide concentrations.

		dGTP (pmol/10^6 cells)	GTP (pmol/mg protein)
Day 0	Median (*n*)	0.18 (10)	4.68 (11)
	IQR	0.14–0.34	3.34–4.80
Day 1	Median (*n*)	0.39 (11)	3.78 (9)
	IQR	0.21–0.72	3.18–5.33
Day 10	Median (*n*)	0.48 (10)	5.02 (10)
	IQR	0.41–0.68	3.77–5.93
Week 8	Median (*n*)	0.37 (9)	4.72 (9)
	IQR	0.23–0.60	3.78–5.34

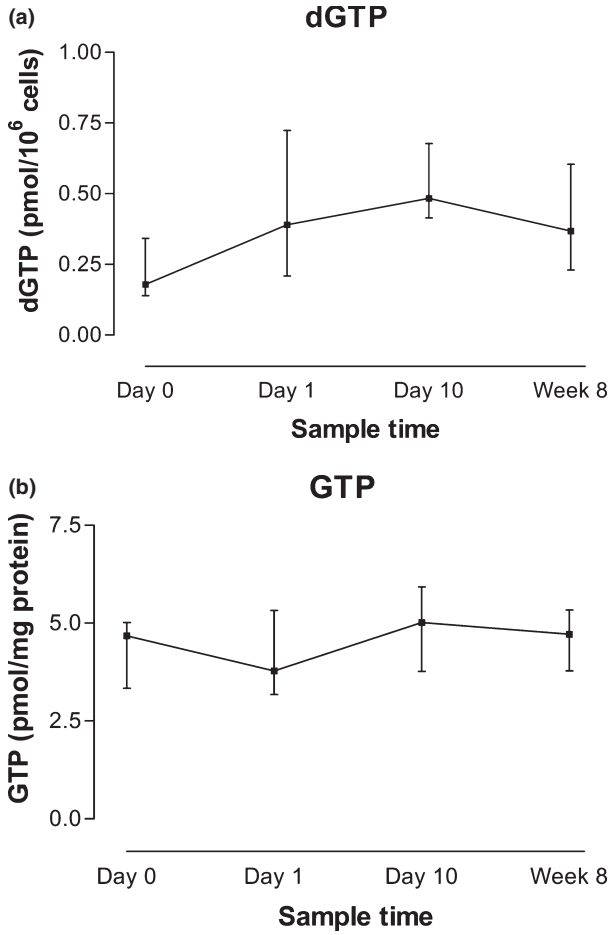

Figure 3 Intracellular concentrations of (a) dGTP and (b) GTP (*x*-axis, sampling time; *y*-axis, intracellular concentrations).

there was a trend towards an increase between day 0 and day 1.

Likewise, there was also no significant change in intracellular GTP levels between baseline and day 1, 10 or week 8 ($P = 0.99$, 0.32 and 0.49; Table 1 and Fig. 3b).

Discussion

Here, we show that treatment of kidney transplant recipients with the IMPDH-inhibitor MMF does not induce depletion of dGTP and GTP in their circulating lymphocyte pool. However, the proliferative capacity of these lymphocytes after polyclonal stimulation *in vitro* was completely inhibited. Although the latter could have been caused by the other immunosuppressive drugs used, the same results were found during treatment of HIV patients with MMF. Indeed, we confirm our previous data, showing inhibited proliferative capacity of lymphocytes without any difference in *in vivo* dGTP concentrations between HIV patients treated with MMF and HIV patients not treated with this drug [7]. Thus, the increased level of immune activation as observed in HIV-infected patients [13] cannot explain that observation.

These findings are in contrast to previously published data from *in vitro* studies suggesting that the immunosuppressive effect of MPA is accomplished by inhibiting guanosine production via the *de novo* pathway of purine synthesis. Jugodzinsky *et al.* [14] demonstrated decreased intracellular GTP levels in lymphocytes from MMF-treated kidney transplant recipients as compared to healthy control individuals. However, in our present study, we measured the nucleotides in kidney transplant recipients before start of MMF treatment and 24 h thereafter. But again, no differences in intracellular dGTP and GTP levels were observed.

Our findings may be explained by the fact that the salvage pathway for the production of dGTP and GTP in lymphocytes might be more important *in vivo* than was observed *in vitro*. In cardiac transplant recipients treated with MMF, it was indeed demonstrated that the salvage pathway was induced, which prevented GTP depletion [15].

Another explanation might be the presence of two isoforms of IMPDH. One isoform (type II), mainly used by activated lymphocytes, is more readily inhibited by MPA than the other isoform (type I) [16]. The proliferation of activated lymphocytes using isoform II might be blocked by MPA resulting in a shift to nonactivated lymphocytes with isoform I on which MPA have less effect. As a consequence there are more lymphocytes without a decrease of dGTP and GTP but, because these cells do not proliferate, the overall thymidine incorporation is low.

A third explanation might be our sampling time. It was recently demonstrated that MPA inhibits IMPDH within 2 h, resulting in a depletion of GTP [17]. After 4 h, the IMPDH activity returned to baseline whereas T-cell proliferation remained below baseline [18]. In our study, the first blood sample after start of MMF treatment was taken just before the next dose of MMF. We therefore may have missed the depletion of dGTP and GTP. Nevertheless, lymphocyte proliferation was inhibited in our patients, suggesting another mechanism by which MMF accomplishes this immunosuppressive effect, such as reduction in the production of cytokines, necessary for an optimal proliferative response [19,20].

Finally, the effects of the other drugs included in the quadruple immunosuppressive drug treatment as given to our kidney transplant recipients might have overruled the action of MMF. Indeed, by co-administration of the other drugs, cells may have been prevented to enter the S-phase of cell division. Although the additional benefit of adding MMF to the immunosuppressive drug regimen after solid

organ transplantation has unequivocally been proven, its mechanism of action might include other points of attack than just inhibition of proliferation. For instance, its ability to alter leucocyte–endothelial adhesion and inhibition of platelet-derived growth factor induced vascular smooth muscle cell proliferation by directly scavenging H_2O_2 may add to overall immunosuppressive capacity [21,22].

In conclusion, we demonstrate that *ex vivo* T-cell proliferation was indeed inhibited in MMF-treated patients, as was shown previously [4]. The suggested mechanism, i.e. depletion of dGTP and GTP, could not be demonstrated *in vivo*, suggesting either another mechanism for inhibiting T-cell proliferation or only early transient decreases in the (d)GTP pool, yet resulting in prolonged inhibition of T-cell proliferation.

Authorship

SUCS: study design, performed study, analysed data, interpretation results and writing manuscript. JMP and IJMTB: study design, interpretation results and writing manuscript. S-LLY: performed immunological part of the study and designed immunological part of the study. JR and ABPVK: designed intracellular GTP part of the study, measurement of intracellular GTP, interpretation of GTP results and writing manuscript. SK and DJB: designed intracellular dGTP part of the study, measurement of intracellular dGTP, interpretation of dGTP results and writing manuscript. FJB: designed immunological part of the study and writing manuscript.

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