Assessment of Mycophenolic Acid-induced Immunosuppression: A New Approach

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Background: Mycophenolic acid (MPA), a metabolite of mycophenolate mofetil (MMF), is an immunosuppressive agent that inhibits inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the ex novo synthesis of GTP. We measured IMPDH activity in peripheral blood mononuclear cells (PBMCs) from MMF-treated patients to evaluate the efficacy of MMF in individual patients.

Methods: IMPDH activity was measured by 3H released from [2,8-3H]IMP that had been formed in the cells from added [2,8-3H]hypoxanthine in PBMCs of 35 renal transplant recipients treated with cyclosporin A and corticoids plus MMF: 2 g (n = 10), 1.5 g (n = 7), 1 g (n = 10), or 0 g (n = 8) per day. An alternative method, based on the capacity of the patients’ sera to inhibit spontaneous proliferation of the CEM cell line, was also analyzed.

Results: The IMPDH activity of PBMCs in transplanted patients was highly variable. For the method based on CEM cell line proliferation: (a) cell proliferation was inhibited only in MMF-treated patients; (b) there was a clear postdose increase in inhibition; (c) inhibition was not affected by other immunosuppressants in vitro or in vivo; (d) inhibition from predose to predose sample was correlated; and (e) when the MMF dosage was <20 mg·kg⁻¹·day⁻¹, two groups of patients were identified, one that maintained a high inhibitory capacity in all dose intervals, and one with periods of low inhibitory capacity.

Conclusions: Measurement of the inhibition of CEM cell line proliferation by sera from MMF-treated patients may be useful for evaluating the relative efficacy of MMF treatment in individual patients, especially those receiving low doses of MMF.

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Mycophenolate mofetil (MMF)4 is an immunosuppressive agent used to prevent rejection. MMF is metabolized to mycophenolic acid (MPA), a potent inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the ex novo synthesis of GTP (1, 2). Lymphocyte clonal expansion is especially dependent on ex novo GTP synthesis. The MPA-induced GTP depletion of lymphocytes inhibits the induction of cyclin D3, a major component of cyclin-dependent kinase, and decreases the degradation of p27kip1, a cyclin-dependent kinase inhibitor, thus arresting the cell cycle in early-to-mid-G1 phase (3).

MMF has a highly variable absorption profile. MMF is hydrolyzed rapidly by esterases in the intestine and blood to release MPA, which crosses the cell membrane (4); however, >99% of MPA remains in the plasma compartment, binding mainly to albumin (5). MPA is metabolized rapidly (2–4 h), mainly to MPA-glucuronide, a metabolite thought to be inactive (6), although not all authors agree (7). Two other minor metabolites have also been described: the 7-O-glucose conjugate of MPA (M1) and the acyl glucuronide conjugate of MPA (M2) (8). M1, at least, appears to be active.

The monitoring of blood concentrations of immunosuppressants is a well-established practice for calcineurin inhibitors and for MMF (9). The monitoring of blood concentrations helps to ensure minimal concentrations (c₁₂₀) and, more particularly, to avoid toxicity, but this monitoring provides only a poor idea of the degree of immunosuppression attained in an individual patient (10). The efficacy of an immunosuppressant probably depends on the interaction between the active form and the target enzyme during the entire dose interval. This interaction depends on a combination of factors such as the absorption pattern, plasma transport proteins, cyto-
plasm binding proteins, the timing of the metabolism of the immunosuppressant, and the activity of metabolites. For these reasons, several methods have been proposed to measure more directly the efficacy of immunosuppressants in individual patients. Some of these methods are based on the measurement of the activity of the putative immunosuppressant’s target enzyme. Based on this rationale, some groups have used measurements of calcineurin activity to assess cyclosporin A (CsA) and FK506 efficacy (11, 12).

A similar approach to the measurement of IMPDH activity has been proposed for monitoring MMF efficacy. For this purpose, a method described by Balzarini and De Clercq (13), which has been used to measure IMPDH in CEM cell lines, has been used by some authors to monitor the IMPDH activity in the peripheral blood mononuclear cells (PBMCs) or in whole blood of MMF-treated animals or patients (14–16).

We used the method of Balzarini and De Clercq (13) to measure IMPDH activity in PBMCs of MMF-treated patients. Because of the high variability of this method and some theoretical considerations presented in the Discussion, we looked for alternative methods of evaluating MMF efficacy. Here we propose an alternative method based on the ability of the sera of MMF-treated patients to inhibit spontaneous proliferation of the CEM cell line (CEM response). We provide data indicating that this marker is not influenced by other immunosuppressants, is related to the dose received and to the MPA blood concentrations, has good reproducibility, and is relatively easy to measure.

Materials and Methods

Reagents

RPMI-1640 was obtained from BioWhittaker. Trichloroacetic and phosphoric acid were purchased from Merck. [2,8-3H]Hypoxanthine (5 mCi) and [methyl-3H]thymidine (5 mCi) were obtained from Movarek Biochemicals. Activated charcoal, thrombin, MPA, and dexamethasone were from Sigma. CsA was kindly supplied by Novartis Farmacéutica S.A. (Barcelona, Spain). OptiPhase “HiSafe” 2 was from Wallac Scintillation Products.

MPA and carbamazepine calibrators were obtained from Sigma-Aldrich. Methanol, HPLC-grade acetonitrile, and glacial acetic acid were purchased from Scharlau. C18 solid-phase extraction columns were from Supelco®.

Source of Specimens

Twenty-seven stable renal transplant recipients receiving 1 g (n = 10), 0.75 g (n = 7), or 0.5 g (n = 10) of MMF twice a day took part in the study (MMF+ group). For analysis purposes, the patients were regrouped on the basis of their dose per kg per day: 10–19 mg · kg−1 · day−1 (n = 9), 20–29 mg · kg−1 · day−1 (n = 12), and >30 mg · kg−1 · day−1 (n = 6). The MMF therapy period ranged from 6 to 14 months. The immunosuppressive drug treatment also consisted of CsA and prednisone. The mean (± SD) age of the patients was 42.5 ± 13.6 years, with a posttransplantation follow-up of 38.5 ± 44.8 months. The control group (MMF−) included eight transplanted patients receiving similar doses of CsA and corticoids, but not MMF, and eight normal healthy controls (NHCs).

To study IMPDH activity, blood samples were collected at the following times: before administration of the dose and 1, 2, 4, and 12 h after administration. All samples were collected in tubes containing heparin as anticoagulant. At the same time, blood was collected in tubes without anticoagulant to obtain sera. To investigate the relationship between plasma MPA concentrations and their biological effect, the inhibition of IMPDH activity was measured in isolated lymphocytes from blood samples at times 0 (predose) and 1, 2, 4, and 12 h after administration.

Isolation and Purification of Lymphocytes

Human PBMCs were obtained from the mononuclear cell layer of the Ficoll-Hypaque gradient. PBMCs were washed twice with phosphate-buffered saline and resuspended with 5 mL of RPMI-1640 containing 100 mL/L heat-inactivated fetal calf serum (FCS). The platelets, monocytes, and residual polymorphonuclear cells were removed by incubation with thrombin (103 IU/L) in a 5-mL disposable syringe containing 200 mg of nylon wool for 30 min at 37 °C in 5% CO2 and 95% humidified air.

The nonadherent cells were collected and centrifuged for 10 min at 400g. The purity of the lymphocytes was >90%.

Cell Line

The CEM cell line was obtained from American Type Culture Collection. CEM is a human T-lymphoblastoid cell line obtained from the peripheral blood buffy coat of a 4-year-old Caucasian female with acute lymphoblastic leukemia. We used the subclone, which we called CEM.2b. We provide data showing that this subclone is resistant to corticoids (prednisone and dexamethasone). This clone will hereafter be designated as CEM. The CEM cells were cultured in RPMI-1640 supplemented with 100 mL/L FCS and gentamicin. The cells were grown at 37 °C in 5% CO2.

Determination of IMPDH Activity in Isolated Lymphocytes and CEM Cells

The IMPDH activity of intact lymphocytes and CEM cells was measured as described previously (13), with some minor modifications.

The enzyme activity was determined by estimating the 3H released from [2,8-3H]IMP, which formed in the cells from added [2,8-3H]hypoxanthine (39.6 Ci/mmol). During the reaction, the 3H atom located on C-2 of the hypoxanthine ring of IMP is replaced by a hydroxyl group. NAD+ serves as the electron acceptor and is reduced to NADH.

The purified lymphocytes and CEM cells were resus-
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Results

IMPDH ACTIVITY MEASUREMENT IN VITRO AND IN VIVO

The measurement of IMPDH activity in our hands was validated; the activity in PBMC and CEM cells was inversely proportional to MPA concentration (Fig. 1).

Because of the good correlation between the MPA concentrations and the IMPDH activity attained in vitro...
the study of transplanted patients was begun. The IMPDH activity of PBMCs from transplanted patients, treated with MMF (n = 27) or not treated with MMF (n = 8), and NHCs (n = 8) was studied. The results showed stable IMPDH activity in the NHCs at 57.5 ± 18.2 dpm/min. On the other hand, predose IMPDH activity in transplanted patients showed a high variability in both groups: 75.4 ± 55.2 dpm/min in the MMF+ group and 84.7 ± 94.7 dpm/min in the MMF− group (Fig. 2).

Although CVs of 5.1% (intraassay) and 9.7% (interassay) were acceptable in any given sample, the correlation between the IMPDH activity measurement in blood samples from predose time (0 h) and new predose time (12 h) in the same transplanted patient was extremely poor (r² = 0.18).

The variability of IMPDH activity between patients was very high at 1, 2, 4 and 12 h post dose: 39.8 ± 41.4, 42.7 ± 49.6, 60.8 ± 41.0, 44.7 ± 54.8 dpm/min, respectively.

CEM PROLIFERATION-BASED APPROACH IN VITRO
Several models were tested. We finally decided to use the spontaneously proliferating CEM cell line as the target for the antiproliferative capability of sera from MMF-treated patients. As expected, the proliferation of the CEM cells was indirectly related to the MPA added to NHS (Fig. 3). In contrast, the proliferation of CEM cells was not affected by NHS with externally added CsA or prednisone alone or in combination (Fig. 3A). Neither CsA nor prednisone modified the inhibition induced by MPA (Fig. 3B). Similar results were obtained with dexamethasone (data not shown).

CEM PROLIFERATION-BASED APPROACH IN VIVO
Spontaneous CEM cell proliferation in the presence of patients’ sera at a 1:1 dilution was tested. The results for patients’ sera were expressed as follows: CEM response = [CEM (cpm) in patients’ sera] × 100/[CEM (cpm) in NHS]. The greater inhibitory capacities of the patients’ sera implied a lower percentage of CEM response. Results indicated that sera from the MMF− group had no inhibitory effect on CEM proliferation. The predose sera inhibited the CEM response to below 40% in all cases when the dose was >19 mg/kg. In the MMF+ (10–19 mg/kg) group, six of nine patients [67%; 95% confidence interval (95% CI), 35–99%] had a predose CEM response below 40%; in three of nine (33%; 95% CI, 1–65%), it was between 40% and 80% (Fig. 4).

The ability of postdose sera at 1, 2, 4, and 12 h to inhibit CEM proliferation was analyzed in the MMF− and MMF+ groups. As expected, it was not possible to demonstrate that CEM response was different at 1, 2, 4, and 12 h compared with the MMF+ group; in contrast, a significant inhibition of CEM response was
seen in the MMF+ group during the posttreatment period (Fig. 5). The CEM response was significantly lower at 1 h (5.1% ± 12.5%; P < 0.001), 2 h (7.8% ± 14.8%; P < 0.001), and 4 h (11.3% ± 12.0%; P < 0.001) compared with 0 h (23.5% ± 18.0%). No difference was seen between 0 and 12 h. The postdose individual profile of each patient is presented in Fig. 6, grouped for clarity purposes according to the predose CEM response: <20% (patients with high predose inhibition; Fig. 6A), >20% but <40% (patients with medium predose inhibition; Fig. 6B), and >40% (patients with low predose inhibition; Fig. 6C). A clear postdose inhibition of CEM response occurred at 1 and 2 h in all but one of the patients. At 12 h, CEM response returned to values similar to predose values. Only one of the patients presented no inhibition at 1 and 2 h post dose (Fig. 6B). This patient deserves special mention because his MPA concentrations were abnormally low (<2 mg/L) at 0, 1, and 2 h, and he had an abnormal MPA peak concentration, 12.3 mg/L, at 4 h. This peak correlated with the peak CEM response inhibition.

Generally speaking, the MMF+ group reached peak MPA concentrations and CEM inhibition at 1 and 2 h, as can be seen in Fig. 7.

The correlation of CEM response from predose measurement to predose measurement (0 and 12 h) was quite good (r² = 0.94). The results obtained by the method using normal human PBMCs activated with PHA + IL-2 or PMA + IL-2 were very similar to those obtained with CEM cells. In fact, the correlation coefficient between CEM response and PMA + IL-2 PBMC response with the predose sera was 0.83, and the correlation coefficient between CEM response and PHA + IL-2 PBMC response was 0.81.

To assess whether CEM response could be predicted by MPA blood concentrations, the capacity of sera to inhibit CEM proliferation at 12 h was correlated with MPA blood concentrations of the MMF+ group. When MPA blood concentrations were 

\[ \text{MPA blood concentrations} \begin{align*} \text{between} & \text{3 mg/L, 11 of 12 patients} & (92\%; 95\% \text{ CI, 77–100\%}) \text{ had a CEM response below 20\%}. \end{align*} \]

On the other hand, when MPA concentrations were <3 mg/L, the patients were distributed into two groups: 6 of 15 patients (40\%; 95\% CI, 15–65\%) had a CEM response above 20\%, and 9 of 15 patients (60\%; 95\% CI, 35–85\%) had a CEM response below 20\% (Fig. 8).

To provide further support for the hypothesis that the inhibitory capacity of sera from MMF-treated patients on
CEM proliferation is related to IMPDH inhibition, CEM cells were cultured for 4 h with patients’ sera (1:1, by volume) collected predose and 1 h post dose. The IMPDH activity of those CEM cells was measured 4 h later (Fig. 9). The results were expressed as: 

\[ \frac{\text{IMPDH activity (dpm/min) of CEM in patients’ sera}}{\text{IMPDH activity (dpm/min) of CEM in NHS}} \times 100 \]

The greater inhibitory capacities of patients’ sera implied lower IMPDH measurements in the CEM. The sera from the MMF group at 0 h, and especially at 1 h post dose, inhibited the IMPDH activity of the CEM cells as expected. Sera from MMF patients did not inhibit CEM IMPDH activity. Sera from the MMF group had a stronger inhibitory effect on CEM IMPDH compared with the MMF group: 97.5% ± 3.8% vs 73.4% ± 29.4% at 0 h (P = 0.001), and 94.8% ± 9.6 vs 56.1% ± 33.9% at 1 h (P < 0.001).

The inhibitory capacity was different in the MMF group at 0 and 1 h (P = 0.02, \( t \)-test for paired samples). As expected, no difference could be detected between 0 and 1 h in the MMF group (P = 0.4).

Discussion

The results presented here confirm previous reports (14, 19) indicating that the measurement of IMPDH activity in the PBMCs of MMF-treated patients is feasible using the technique described by Balzarini and De Clercq (13). Langman et al. (19) found no difference in IMPDH activity in PBMCs between MMF-treated patients and controls. A difference was found only when IMPDH activity was measured in whole blood. In our data, high variability existed between transplanted patients, especially in the non-MMF-treated group, and from predose sample to predose sample in the same patient. In our opinion, this variability and the previously published data of Langman et al. (19) jeopardize the routine use of this technique in the measurement of MMF efficacy in individual patients.

Because of the high variability detected in the patients and the poor stability of the IMPDH activity in individual patients, we considered a new approach. We here propose another method based on the capacity of serum to inhibit proliferation of the CEM cell line. We show here that this method (a) has a direct relationship with externally added MPA in NHS; (b) is not altered by the presence of other immunosuppressants such as CsA or prednisone; and (c) has a good correlation from predose sample to predose sample. Furthermore, the CEM response identifies a subgroup of patients who have less inhibitory capacity in the low-dose MMF group (10–19 mg·kg\(^{-1}\)·day\(^{-1}\)). It also identifies a group of patients who despite having MPA blood concentrations <3 mg/L, have a good capacity to inhibit clonal expansion in vitro.

As far as we know, only two groups had published data concerning the measurement of IMPDH in MMF-treated patients (14, 15), probably because of difficulties in the implementation of this technique. Our results indicate that the measurement of IMPDH activity has a high variability in transplanted patients, even without

Fig. 7. Correlation between MPA concentrations and CEM response in the MMF-treated patients.

Results are expressed as the percentage of cpm compared with CEM cells cultured with NHS (left y axis) and mg/L MPA (right y axis).

Fig. 8. Correlation between the capacity of patients’ sera to inhibit CEM proliferation at 12 h (%CEM 12 h), measured as described in Materials and Methods, and MPA blood concentrations (12 h) measured by HPLC.

Fig. 9. IMPDH activity in CEM cells cultured with patients’ sera.

CEM cells were cultured for 4 h with patients’ sera (1:1 final dilution) collected predose and 1 h postdose from patients treated with MMF (MMF+) and patients not treated with MMF (MMF−). IMPDH activity was measured using \([^{3}H]\)hypoxanthine. Results are expressed as the percentage of IMPDH activity compared with CEM cells cultured with NHS.
MMF treatment, and has poor stability from predose sample to predose sample.

From a theoretical point of view, the direct measurement of IMPDH in PBMCs or in whole blood as an indicator of MMF efficacy has some possible drawbacks: (a) The majority of PBMCs are resting lymphocytes that contain mainly IMPDH-I. In contrast, activated lymphocytes contain mainly IMPDH-II (20–22), which is four times more sensitive to MPA than IMPDH-I (23). (b) The activated lymphocytes are only a very small proportion of PBMCs, but they are the relevant ones for rejection. (c) Ninety-nine percent of MPA is retained in the plasma compartment, mainly bound to albumin (5, 6), and <1% is located inside the mononuclear cell fraction. On the basis of these considerations, it can be hypothesized that clinical MMF efficacy is related mainly to the capacity of the plasma to maintain a constant release of “free” MPA (or an active metabolite) to activated lymphocytes. The IMPDH activity of nonactivated cells can create a background noise that jeopardizes the utility of this marker because of the poor sensitivity to MPA of IMPDH-I and to the irrelevance of IMPDH-I to rejection in this majority population.

The method we propose here has its base in the capacity of serum to inhibit CEM proliferation. We have shown that results obtained with this method directly relate to the MPA externally added to the sera and that the method is not affected by other immunosuppressants such as CsA or corticoids, even at doses as high as 400 µg/L. This means that the method does not measure the overall concentration of immunosuppressants in the patient but has the advantage of differentiating the role of MPA when it is used in combination with others drugs. We also tested other models such as (PHA + IL-2)- or (PMA + IL-2)-activated PBMCs. Although both have a good correlation with CEM response, in general terms (PHA + IL-2)-activated PBMCs were partially affected (~20% inhibition) by in vitro exogenously added CsA at 200 µg/L. (PMA + IL-2)-activated PBMCs, on the other hand, were not affected by CsA, but it was not possible to measure IMPDH activity in these cells. Apart from these considerations, the CEM model presents less variability than the others. For all of these reasons, we chose the CEM response method. The use of the same target cell for all patients has the disadvantage of not measuring patient variability in the last intracellular steps, but it reduces the technical variability, allowing a better comparison between patients under different immunosuppression regimes while measuring the ability of an active form of the drug to be released from plasma proteins in a form able to cross the cell membrane and interact with the target enzyme, having the desired effect on lymphocytes.

Data from the sera of the MMF− group of patients showed no significant inhibition of CEM response, confirming that this marker is not affected by other immunosuppressants in vivo. Some patients had values >100% because the proliferation was always referred to a standard serum and those patients had proliferations higher than that found in the standard serum. On the other hand, a clear inhibition was seen in the sera of the MMF+ group, both predose and postdose.

The predose sera values indicated that the sera of patients receiving >20 mg/kg have a inhibitory effect on CEM response during the entire dose interval, maintaining a CEM response below 40% in all cases. In contrast, the patients receiving <20 mg/kg could be divided into two groups: 67% with a CEM response below 40% and 33% with a response above 40%. We do not know whether 40% indicates a sufficient concentration of immunosuppression to control rejection, but we can hypothesize that in relative terms those patients with a low capacity to inhibit CEM response in predose intervals are probably less immunosuppressed than those with a high inhibitory capacity during the entire dose interval.

Results for 1- and 2-h postdose sera indicate that the inhibitory effects of sera from all but one patient on the CEM response increased after the MMF dose, maintaining CEM response below 20% in 25 of 27 cases, indicating a clear postdose effect. The patient whose serum did not inhibit the cell response also had an abnormal blood concentration pattern, with low MPA concentrations at 1 and 2 h and a peak MPA concentration at 4 h. Individual patient data also indicated that patients with a low inhibitory capacity at 0 h had a similar response at 12 h. These data were confirmed by the good correlation coefficient (r² = 0.94) between both predose samples (0 and 12 h).

Patients with MPA concentrations >3 mg/L correlated in almost all cases (11 of 12; 92%) with a predose CEM response below 20%. In patients with MPA concentrations <3 mg/L, CEM response was distributed almost equally between below 20% (60% of patients) and above 20% (40% of patients). These data indicate that although high MPA concentrations are an indicator of good inhibition of CEM response, low concentrations can be just as efficient as high concentrations in some, but not all, patients. The variability in MPA metabolism, the activity of MPA metabolites, or MPA binding could be the reason. Measurement of CEM response could be a good method for identifying patients with a high capacity to inhibit cell proliferation although with low MPA concentrations.

Sera from the MMF+ group but not from the MMF− group inhibited the IMPDH activity of the CEM cell, and these results seem to favor the hypothesis that the effects seen in the patients’ sera are certainly related to the MPA putative target enzyme and not to any other phenomena. It can be argued that the inhibition of IMPDH activity is not complete and that a residual 50% activity remains in the CEM cells at the same time (1 h) that the CEM response is highly inhibited. In our opinion, this could be only a methodological problem related to the number of CEM cells used (75 000), the small volume of serum used (100 µL), or the short incubation time (4 h). Higher inhibition could probably be seen by reducing the number
of target cells or increasing the incubation time to 24 h (as in proliferation assays).

A hypothetical interference in the technique we propose could be produced by the presence of anti-HLA antibodies reacting with CEM cells. In our group of patients, only four had panel-reacting antibodies, and three of four had a predose CEM response above 40%, indicating that these antibodies had no inhibitory effect in our study. A possible explanation for these data could be the fact that human complement is relatively inefficient in killing human cells. For this reason, rabbit complement is needed in the panel-reacting antibody test to kill panel cells. Despite this, we cannot rule out the possibility that some antibodies from hypersensitized patients could create an interference via CEM-reacting anti-HLA antibodies.

On the basis of the data presented here, we hypothesize that the ability of sera from MMF-treated patients to inhibit CEM response could be useful for evaluating the efficacy of MMF treatment in individual patients. We cannot yet predict which level of CEM response suppression will correlate with rejection-free kidney survival, but an approach can be proposed in relative terms. For this reason, we postulate that those patients maintaining a predose CEM response above 40% have a level of immuno-suppression relatively lower than the ones who have a predose CEM response below 40%. For this reason, the MMF dose should be increased in these patients to attain a relative immuno-suppression equivalent to the one found in the rest of the MMF-treated patients. Moreover, those patients who are able to maintain a predose CEM response below 20% with low doses of MPA could be considered, relatively speaking, as being as well suppressed as patients receiving higher doses, at least as far as the sensitivity of the technique can predict. However, the above statement must obviously be confirmed through correlation with rejection episode data in wider studies.

Putative optimal doses of MMF often have to be reduced on the basis of individual side effects. In these cases, it becomes necessary to evaluate the individual efficacy of these "suboptimal" doses. We postulate that measurement of the predose capacity of serum to inhibit CEM response could be helpful in this task.

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