IMP-dehydrogenase inhibition in human lymphocytes and lymphoblasts by mycophenolic acid and mycophenolic acid glucuronide

Andrea Griesmacher,1,2* Günter Weigel,2 Gernot Seebacher,2 and Mathias M. Müller1

Inosine 5′-monophosphate dehydrogenase (IMP-DH) activities were measured in human lymphocytes (exhibiting type I IMP-DH activity) and human lymphoblasts (exhibiting type II IMP-DH activity) in the presence of various amounts of mycophenolic acid (MPA) (0–20 μmol/L) and MPA glucuronide (MPAG) (0–200 μmol/L). Moreover, the influences of human serum albumin (HSA) and human plasma on the MPA- and MPAG-mediated effects were investigated. In the presence of water, 2.5 μmol/L MPA decreased the IMP-DH activity measured in lymphocytes by 60%, whereas in lymphoblasts a 80% inhibition was detectable. In the presence of ≥10 μmol/L MPA, lymphocytic as well as lymphoblastic IMP-DH activities were reduced in a similar manner. The concentration of MPAG required for 50% inhibition was for both cell types ≥25 μmol/L and <50 μmol/L, respectively. MPAG (200 μmol/L) reduced lymphocytic as well as lymphoblastic IMP-DH activity by ~80%. With 100 g/L HSA or human plasma as diluent, the inhibitory effects of MPA and MPAG were significantly (P <0.05) diminished, whereas HSA concentrations ≤25 g/L only slightly influenced the inhibition of IMP-DH activity by MPA and MPAG. In summary, it can be clearly demonstrated that not only MPA but also MPAG contributes to the inhibition of both IMP-DH isoenzymes, which might be relevant for the immunosuppressive properties of mycophenolate mofetil in transplant patients.

Mycophenolic acid (MPA) possesses immunosuppressive, anticancer, antibacterial, and antiviral properties [1–4] and mediates its effects by noncompetitive reversible inhibition of the enzyme inosine 5′-monophosphate dehydrogenase (IMP-DH; EC 1.1.1.205) [5]. IMP-DH catalyzes the NAD1-dependent oxidation of IMP to xanthosine 5′-monophosphate (XMP), an intermediate metabolite in the synthesis of GTP, and is considered to be the rate-limiting enzyme in the de novo guanylate biosynthetic pathway [6]. Human IMP-DH exists as two isoforms derived from different genes, designated type I and type II [7,8]. Type I is constitutively expressed in normal leukocytes, whereas type II is up-regulated in neoplastic and replicating cells [9,10]. With purified recombinant proteins type II IMP-DH is 4.8-fold more sensitive to MPA than type I [11].

Up to now, it has not been clear which IMP-DH isoform is the more important therapeutic target of MPA [12] when used for immunosuppression in humans. Early reports indicated that only type II is up-regulated during proliferation of lymphatic and leukemic cells [9,10,13,14], whereas recent studies with T cells demonstrated similar increases in the mRNA concentrations of both isoforms after stimulation [15].

Mycophenolate mofetil (MMF) (Syntex), an ester prodrug of MPA, was recently approved for the prevention of acute allograft rejection [16,17]. The biochemical action of MPA causes dose-dependent inhibition of DNA synthesis in lymphocytes, leading to impairment of the cellular immune response.

MMF, which is nowadays administered to transplant recipients as adjunctive therapy to cyclosporin A and corticosteroids, is in vivo hydrolyzed to MPA. It is further metabolized to a single glucuronidated metabolite MPA glucuronide (MPAG), which is then excreted in urine [18]. Transplant patients at our department receiving MMF...
exhibit ~50-fold higher plasma concentrations of MPAG than of MPA.

Papers dealing with the inhibitory effects of MPAG on IMP-DH activity are contradictory. While Lee et al. [18] did not find any effect of MPAG, Langman et al. [19] observed a weak enzyme inhibition by MPAG in intact lymphocytes.

This study was undertaken to investigate the effects of clinically relevant concentrations of MPA and MPAG on the type I and type II IMP-DH activity. For this purpose human lymphocytes (exhibiting type I isoform) and human lymphoblasts (exhibiting type II isoform) were incubated with various amounts of MPA and MPAG and IMP-DH activities were measured with a nonradioactive method.

In addition, the influence of human serum albumin (HSA) and human plasma on the MPA- and MPAG-mediated effects were investigated, because the pharmacological activity of MPA is suggested to be a function of unbound drug concentration [20].

**Materials and Methods**

**CHEMICALS AND SPECIMENS**

MPAG was a generous gift from Syntex Research. A purity >99.8% was confirmed by nuclear magnetic resonance (NMR) as well as by mass spectroscopy. MPA, NAD\(^+\), IMP, XMP, GMP, and AMP were from Sigma Chemical Co.; Tris, NaH\(_2\)PO\(_4\), KCl, NAD\(^+\), HClO\(_4\), NaH\(_2\)CO\(_3\) from Merck; and HSA from Biotest Pharma. Total protein was measured with a kit obtained from Bio-Rad with the protein–dye-binding method (Bradford). Ficoll-Paque was from Pharmacia Fine Chemicals. RPMI 1640, fetal bovine serum (FBS), l-glutamine, penicillin/streptomycin solutions, and Dulbecco’s PBS (DPBS) were from Gibco Labs.; Hank’s balanced salt solution (HBSS) was from BioWhittaker. Heparinized whole blood was obtained from healthy volunteers, who did not receive any drugs, for the preparation of lymphocytes and the plasma pool. MOLT-3 cells, which are human peripheral lymphoblasts, were obtained from ATCC.

**PROCEDURES**

**Isolation of lymphocytes.** Whole blood was separated by density-gradient centrifugation in Ficoll-Paque; lymphocytes were isolated from peripheral blood mononuclear cells by centrifugal elutriation in HBSS medium containing 10 mL/L FBS [21]. Cell viability, assessed by trypan blue exclusion, varied between 95% and 98%. About 96% to 99% of cells were scored as lymphocytes as determined by flow cytometric scattergrams. After resuspending the cells, aliquots of cell suspensions were used for cell counting in an automated counter (Coulter). For the experiments, cells were diluted with deionized and distilled water to yield 2.5 × 10\(^6\) ± 1 × 10\(^6\) cells/L.

**Cultivation of lymphoblasts.** MOLT-3 cells were cultivated with RPMI 1640, 100 mL/L FBS, 100 kIU/L penicillin, 100 mg/L streptomycin, and 2 μmol/L l-glutamine. Cell cultivation was carried out in a humidified incubator set at 37 °C and 5% CO\(_2\). For the experiments cells were washed twice with DPBS and diluted with deionized and distilled water to a final concentration of 2.5 × 10\(^8\) ± 9 × 10\(^7\) cells/L.

**Determination of activity.** Methanol was used as vehicle for preparing 5 mmol/L stock solutions of MPA and MPAG, which were diluted to yield final concentrations of 0–40 μmol/L and of 0–400 μmol/L, respectively, with deionized and distilled water; 5–200 g/L HSA or human plasma containing 81 g of protein/L (48 g/L of the total protein was albumin) was also used. Cells were cracked by freezing and thawing twice. Cell lysate (30 μL) was preincubated with 30 μL of various amounts of MPA or MPAG at 37 °C for 30 min before the determination of enzyme activity. Cell lysates with equal amounts of water, HSA, or human plasma served as controls.

IMP-DH activity was measured by means of HPLC by using a modified procedure described by Montero et al. [22]. Assays were carried out at 37 °C in a total volume of 180 μL. The incubation buffer consisted of 80 mmol/L NaH\(_2\)PO\(_4\), 200 mmol/L KCl, 500 μmol/L NAD\(^+\), and 1000 μmol/L IMP (pH 7.5). The reaction was started by addition of 60 μL of cell lysate and carried out for 2.5 h for lymphocytes and for 1.5 h for lymphoblasts. The incubation was stopped by addition of 35 μL of HClO\(_4\). After centrifugation 180 μL of supernatant was neutralized with 18 μL of KH\(_2\)PO\(_4\).

IMP, XMP, GMP, and AMP were separated by injecting 100 μL of the neutralized supernatant onto a CNU-010 column (Chemcon) with a K\(_2\)HPO\(_4\) gradient. Buffer A consisted of 0.015 mol/L K\(_2\)HPO\(_4\) (pH 3.45), buffer B of 0.5 mol/L K\(_3\)HPO\(_4\) (pH 3.45). A linear gradient rising from 0% B to 50% B in 8 min was used with a total run time of 28 min and an equilibrium delay of 5 min. The flow rate was 1.2 mL/min. The intraassay (n = 7) and interassay (n = 20) CVs for the measurement of IMP-DH activity were 10.9% and 16.4%, respectively, for 2.7 nmol/10\(^6\) lymphocytes per hour and 7.6% and 9.8% for 11.9 nmol/10\(^6\) lymphoblasts per hour, respectively.

Under the test conditions, only formation of XMP was detected and was therefore alone considered in the calculation of IMP-DH activity (given as μmol/10\(^6\) cells per hour). The amount of formed XMP was determined by the ratio of its peak area in relation to XMP calibrators measured under the same conditions. The linear range for XMP was between 15 and 1000 μmol/L. Interassay CV for the XMP detection was <11% at 15 μmol/L XMP. Methanol in the same concentrations as it had as vehicle has no influence on activity.

**STATISTICAL ANALYSIS**

All experiments were performed in duplicate on at least three separate occasions. Differences between the groups were tested for statistical significance by using analysis of
variance. All calculations were carried out with the statistical software package SAS/STAT [23]. A P-value of <0.05 was considered statistically significant. Values are expressed as mean ± SD.

**Results**

Comparison of IMP-DH activity measured in lymphocytes and lymphoblasts in the presence of water, HSA, or plasma. Table 1 shows IMP-DH activities of the control experiments given as both nmol/10⁶ cells per hour and nmol/mg protein per hour. Lymphocytes (10⁶) were equivalent to 10.5 ± 1.0 μg protein, 10⁶ lymphoblasts to 29.3 ± 3.7 μg protein. IMP-DH activity measured in lymphocytes was decreased by 50% in the HSA group and 30% in the plasma group compared with the water group. In the case of lymphoblasts no differences in enzyme activity were seen between the three groups. For this reason only the matching control group was used as 100% values in the following experiments.

Comparison of MPA-induced inhibition of IMP-DH activity measured in lymphocytes and lymphoblasts in the presence of water, HSA, or plasma. In the presence of water, 2.5 μmol/L MPA decreased lymphocytic IMP-DH activity by 60% (Table 2), whereas in lymphoblasts even an 80% inhibition was detectable (Table 3). Concentrations of ≥10 μmol/L MPA inhibited the enzyme activity by ~85% in both cell types. In the presence of HSA or plasma the inhibitory effects of MPA were diminished in lymphocytes as well as in lymphoblasts.

Comparison of MPAG-induced inhibition of IMP-DH activity measured in lymphocytes and lymphoblasts in the presence of water, HSA, or plasma. In the presence of water MPAG showed very similar effects on IMP-DH activity measured in lymphocytes (Table 4) and lymphoblasts (Table 5). The concentration of MPAG required for 50% inhibition was for both cell types >25 μmol/L and <50 μmol/L, respectively. MPAG (200 μmol/L) reduced lymphocytic as well as lymphoblast.

### Table 1. Comparison of IMP-DH activities measured in lymphocytes (type I IMP-DH) and lymphoblasts (type II IMP-DH).

| Cell lysate (30 μL) preincubated with 30 μL of: | Lymphocytes | | Lymphoblasts | |
|-------------------------|-------------|-------------|-------------|
|                         | nmol/10⁶ cells per hour | nmol/mg protein per hour | nmol/10⁶ cells per hour | nmol/mg protein per hour |
| Water                   | 2.7 ± 0.5 | 267 ± 36 | 11.9 ± 0.9 | 396 ± 35 |
| 200 g/L HSA             | 1.3 ± 0.2* | 129 ± 19* | 12.7 ± 1.6 | 429 ± 42 |
| Plasma                  | 1.9 ± 0.6 | 178 ± 29 | 11.5 ± 3.0 | 395 ± 58 |
| * Statistically significant differences (P < 0.05): water vs HSA. |

### Table 2. MPA-induced inhibition of IMP-DH activity measured in lymphocytes (type I IMP-DH) in the presence of water, HSA, or plasma.

<table>
<thead>
<tr>
<th>Cell lysate (30 μL) preincubated with 30 μL of:</th>
<th>MPA, μmol/L</th>
<th>0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>41 ± 5</td>
<td>30 ± 8</td>
<td>15 ± 6</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>IMP-DH, % of control</td>
<td></td>
<td>100</td>
<td>68 ± 11</td>
<td>49 ± 13*</td>
<td>27 ± 4*</td>
<td>22 ± 5*</td>
</tr>
<tr>
<td>200 g/L HSA</td>
<td></td>
<td>100</td>
<td>71 ± 16</td>
<td>46 ± 11*</td>
<td>31 ± 8*</td>
<td>28 ± 4*</td>
</tr>
<tr>
<td>* Statistically significant differences (P &lt; 0.05): water vs HSA or water vs plasma.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. MPA-induced inhibition of IMP-DH activity measured in lymphoblasts (type II IMP-DH) in the presence of water, HSA, or plasma.

<table>
<thead>
<tr>
<th>Cell lysate (30 μL) preincubated with 30 μL of:</th>
<th>MPA, μmol/L</th>
<th>0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
<th>20.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>20 ± 3</td>
<td>19 ± 3</td>
<td>16 ± 3</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>IMP-DH, % of control</td>
<td></td>
<td>100</td>
<td>45 ± 10*</td>
<td>41 ± 8*</td>
<td>35 ± 6*</td>
<td>25 ± 6*</td>
</tr>
<tr>
<td>200 g/L HSA</td>
<td></td>
<td>100</td>
<td>54 ± 16*</td>
<td>39 ± 11*</td>
<td>33 ± 8*</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>* Statistically significant differences (P &lt; 0.05): water vs HSA or water vs plasma.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
as lymphoblastic IMP-DH activity by ~80%. HSA and plasma again reduced the inhibitory effect of MPAG in both cell types significantly.

Effects of increasing amounts of HSA on MPAG-induced inhibition of IMP-DH activity measured in lymphoblasts. Fig. 1 shows the results of the IMP-DH activity measured in the presence of different concentrations of MPA and HSA. The presence of ≥50 g/L HSA in the cell lysate/MPA mixture strongly diminished the MPAG-induced enzyme inhibition and increased the total MPA concentration required to induce a certain degree of enzyme inhibition. HSA concentrations ≥25 g/L had no influence on the inhibition of IMP-DH activity by MPA.

Effects of increasing amounts of HSA on MPAG-induced inhibition of IMP-DH activity measured in lymphoblasts. Fig. 2 shows the results of lymphoblastic IMP-DH activity measured in the presence of different concentrations of MPAG and HSA. When the cell lysate/MPAG mixture contained 0–25 g/L HSA, 50 μmol/L MPAG was sufficient to reduce the IMP-DH activity by >50%. In the presence of ≥50 g/L HSA the concentration of MPAG required for 50% inhibition of enzyme activity was higher than the concentrations tested.

**Discussion**

This study was undertaken to investigate the influence of clinically relevant concentrations of MPA and MPAG on the IMP-DH activity in human lymphoblasts and human lymphocytes. Since it is not clear which IMP-DH isoform is the more important therapeutic target of MPA [12], both cell types were tested. In lymphocytes type I isoform is predominant, while lymphoblasts possess more of the type II isoenzyme [9, 10]. Under our test conditions IMP-DH activity in lymphoblasts was with regard to the cell count sixfold and regarding the protein content two-fold higher than in lymphocytes.

The concentrations of MPA and MPAG used for the in vitro experiments were based upon the blood trough concentrations (MPA 4.7 ± 6.1 μmol/L, range 0.5–15.2 μmol/L; MPAG 233 ± 175 μmol/L, range 46–551 μmol/L; n = 73) measured in heart- or lung-transplanted patients receiving 2 g/day MMF at our department. The inhibition of IMP-DH activity was more pronounced in lymphoblasts than in lymphocytes at MPA concentrations ≥5 μmol/L. In the presence of ≥10 μmol/L MPA, lymphocytic as well as lymphoblastic IMP-DH activities were reduced in a similar manner. These results could be explained by the fact that MPA is reported to inhibit type II isoenzyme with a 4.8-fold lower \( K_i \) than type I [11]. In contrast, MPAG reduced lymphoblastic as well as lymphocytic IMP-DH activity to the same extent. To our knowledge, no detailed studies of the possible pharmacological effects of MPAG have been published. Studies dealing with the effects of MPAG on IMP-DH activity are contradictory: Lee et al. [18] reported that MPAG is pharmacologically inactive but may be hydrolyzed in vivo to form free MPA, whereas Langman et al. [19] observed 74% enzyme inhibition in intact cells at 230
μmol/L MPAG. In our test system the concentration of MPAG (diluted with water) required for 50% inhibition was >25 μmol/L and <50 μmol/L for both lymphocytes and lymphoblasts, respectively. Under the same conditions 200 μmol/L MPAG reduced lymphocytic as well as lymphoblastic IMP-DH activity by ~80%. To exclude an unspecific hydrolysis of MPAG to MPA [24] under our experimental conditions, MPA was measured by means of HPLC at the end of the whole incubation procedure (data not shown). Since not even traces of MPA were detected in specimens containing 25–200 μmol/L MPAG, it can be excluded that the inhibiting effects observed in presence of MPAG are attributable to in vitro hydrolysis of MPAG.

In the Langman study 6.2–15.6 μmol/L MPA was required for a 50% inhibition [19], whereas in our experiments already 5.0 μmol/L MPA (diluted with HSA or plasma) was enough to attain the same effects in lymphocytes. Moreover, Langman et al. reported about a 74% enzyme inhibition at 230 μmol/L MPAG, whereas in our study 200 μmol/L MPAG (also diluted with HSA or plasma) led to a 45% inhibition of lymphocytic IMP-DH. The differences in the extent of inhibition might be due to the fact that we used lysates for the incubation experiments, in contrast to Langman et al., who used whole blood and a rather unspecific assay for IMP-DH activity measurement.

Recently, Nowak and Shaw showed that MPA binds to HSA [20]. Since the pharmacological activity of MPA is suggested to be a function of unbound drug concentration, we tested the influence of increasing amounts of HSA on the MPA- and MPAG-induced inhibition of IMP-DH activity. The presence of ≥50 g/L HSA in the cell lysate/MPA or cell lysate/MPAG mixture strongly diminished the MPA- and MPAG-induced enzyme inhibition and increased the total MPA and MPAG concentration required to produce a certain degree of enzyme inhibition.
inhibition. Our data emphasizes the hypothesis that unbound MPA (and also MPAG) are responsible for the pharmacological effects.

Taking therapeutic MPA and MPAG blood concentrations into consideration, our data clearly show that MPA and its metabolite exhibit inhibitory effects on both type I and type II IMP-DH, whereby MPAG acts via a noncompetitive mechanism as was described for MPA (data not shown). Thus, both MPA and MPAG contribute to the immunosuppressive effect in vivo. Their transport into the cells is not rate-limiting, since Slingerland et al. [25] inhibited the complete flux from hypoxanthine into guanine ribonucleotides in intact cells by preincubating the cells with 10 μmol/L MPA for 15 min. Thus, the presented study could be helpful for monitoring the biochemical effects of MPA and MPAG by the measurements of IMP-DH with lysates of lymphocytes or lymphoblasts.

Recent reports indicate that MPA inhibits IMP-DH by acting as a replacement for the nicotinamide portion of the nicotinamide adenine dinucleotide cofactor and a catalytic water molecule [12]. Whether the same mechanism of inhibition is also valid for MPAG needs to be further investigated. Nevertheless, plasma MPAG concentrations should be taken into consideration more during therapy of transplant patients with MMF, since it not only contributed to the therapeutic but also to the unwanted side effects.

In summary, we clearly demonstrated that MPA and MPAG contribute to the inhibition of both IMP-DH isoenzymes. This inhibition is partially neutralized by addition of HSA or plasma.

We thank Anneliese Nigisch for her excellent and competent technical assistance and Andreas Spittler for the preparation of human lymphocytes.

References