Pharmacodynamic Assessment of Mycophenolic Acid-Induced Immunosuppression by Measuring IMP Dehydrogenase Activity

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Pharmacodynamic monitoring of the biological effect of immunosuppressive drugs provides an alternative to traditional therapeutic drug monitoring. We chose this method to investigate mycophenolic acid (MPA), an immunosuppressive drug that mediates its effect by inhibition of IMP dehydrogenase (IMPDH), a key enzyme in the de novo biosynthesis of purines. Using an assay developed for measuring IMPDH activity in whole blood, we found the concentration of MPA required for 50% inhibition of enzyme activity to be in the range of 2.0–5.0 mg/L for both human and rabbit blood. The amount of enzyme activity in whole blood depended on the concentration of the leucocytes, was unaffected by the type of anticoagulant used, and was stable in blood specimens stored for as long as 48 h at 4°C. An inverse relationship was found between plasma MPA concentrations and IMPDH activity in rabbits administered a single dose of RS-61443, the prodrug of MPA. Maximal inhibition of IMPDH activity (by ~60%) occurs at peak concentrations of MPA; as the concentration of the drug decreases postdose, the enzyme activity gradually increases with little or no inhibition being observed 24 h postdose.

Indexing Terms: monitoring therapy/RS-61443/prodrugs/rabbit models/enzyme inhibition assay/leukocytes

RS-61443 (RS) is a morpholinoethyl ester of mycophenolic acid (MPA) (1). A product of several penicillium species (1–3), MPA possesses antibacterial, antifungal, antiviral, and immunosuppressive properties. RS can be considered a prodrug because its immunosuppressive activity is expressed only after hydrolysis to MPA (1–3); indeed, RS was formulated to enhance the bioavailability of MPA (1). MPA mediates its effect by inhibiting inosine monophosphate dehydrogenase (IMPDH; EC 1.2.1.14) (1–3), an enzyme that catalyzes the oxidation of inosine monophosphate (IMP) to xanthine monophosphate (XMP), an intermediate metabolite in the synthesis of guanosine triphosphate (GTP). IMPDH is a key enzyme in the de novo biosynthesis of purine nucleotides, particularly guanosine monophosphate (GMP) (2–4). Lymphocytes rely on the de novo purine synthesis pathway for nucleotides necessary for DNA synthesis; other cells can also rely on the salvage pathway (2–5). Therefore, the action of MPA results in the depletion of the nucleotide pool in these cells and subsequent dose-dependent inhibition of DNA synthesis. The cellular depletion of GMP, and similarly of GTP, also retards the transfer of saccharide residues to glycoproteins, especially those expressed on adhesion molecules of lymphocytes during the immunorejection process (6). MPA also inhibits proliferation of B cells, thereby preventing primary polyclonal and antigen-specific secondary antibody responses to antigens, as well as the natural antibodies that mediate hyperacute rejection in xenotransplantation (7, 8).

RS has been efficacious in prolongation of cardiac, renal, and islet allografts in several animal models, including mice, rats, dogs, and humans (9–12), and numerous clinical trials with the drug are underway. Although a few reports have been published regarding the pharmacokinetics of RS in animals and humans (13, 14), little has been reported on the therapeutic range for the drug, and we do not know at present whether therapeutic monitoring of MPA will be necessary to optimize immunosuppressive efficacy or to minimize toxicity.

Therapeutic monitoring of immunosuppressive drugs traditionally involves the measurement of drug concentrations in whole blood or plasma (15, 16). However, assessing the therapeutic concentrations of individual drugs in patients receiving multiple immunosuppressive drug therapy is difficult. An alternative approach involves measuring the effect of the drug, not its concentration, to assess the state of immunosuppression. Given that MPA inhibits IMPDH, pharmacodynamic monitoring of the degree of inhibition of the enzyme may provide a better indicator of immunosuppression than will the drug concentrations in plasma. We report here the establishment of an assay for measuring IMPDH in whole blood and describe the relationship between MPA concentrations and the degree of inhibition of this enzyme.

Materials and Methods

Drugs and Specimens
RS, MPA, and the glucuronide metabolite of MPA (MPAG) were obtained as gifts from Syntex (Palo Alto, CA). Whole blood was collected from apparently healthy human volunteers into tubes containing heparin, EDTA, or acid–citrate–dextrose anticoagulant. The procedures followed were in accordance with the ethical standards of the University of Alberta. Whole blood was also collected via the marginal ear vein from
New Zealand White rabbits given a single intravenous dose of RS (40 mg/kg body wt.).

Procedures

Isolation of lymphocytes. Lymphocytes were separated from blood as previously described (17, 18). Pooled fresh whole blood was layered over an equal volume of Histopaque 1077 (Sigma Diagnostics, St. Louis, MO) and centrifuged for 30 min at 400g. The resulting plasma and mononuclear layers were removed and washed twice with RPMI medium (Gibco Labs., Burlington, ON, Canada). The platelets and monocytes were removed from the suspension by adding a drop of Thrombostat (5000 IU/L; Parke-Davis, Scarborough, ON) and then centrifuged for 60 s at 400g. The purity of the lymphocytes in the supernate, as determined microscopically, was > 95%. For experiments other than those specified, the isolated lymphocytes were incubated for 30 min in RPMI medium before determination of enzyme activity as described below.

Determination of IMPDH activity in isolated lymphocytes. We measured the activity of IMPDH in intact lymphocytes by a modification of a procedure described by Balzarini and De Clercq (19). Briefly, the enzyme activity is determined by estimating the 3H released from [2,8-3H]IMP that has been formed in the cells from added [2,8-3H]hypoxanthine (Hx, 15 Ci/mol) or [2,8-3H]inosine (Ino, 30 kCi/mol) both from Moravek Pharmaceuticals, Brea, CA. During the reaction, the tritium atom located on C-2 of the hypoxanthine ring of IMP is replaced by a hydroxy group. NAD+ serves as the electron acceptor and is reduced to NADH. The rate of catalysis with the 3H substrate is less than that with nonlabeled substrate because of the tritium isotope effect (20).

To 300 μL of lymphocyte at a concentration of 2.5 × 10^7/L, we added 100 μL (10 μCi) of radiolabeled Hx or Ino. After 0, 10, 20, and 30 min, we removed 100-μL aliquots and mixed them with 500 μL of freshly prepared cold suspension of 100 g/L activated charcoal in 50 g/L trichloroacetic acid. We then centrifuged the samples at 1300g for 10 min and analyzed 200 μL of the supernate for radioactivity by scintillation counting (Minaxiβ Tri-carb 4000 series; Canberra Packard, Toronto, Canada), using a quench-corrected counting program. The amount of spontaneous liberation of 3H, measured with each analysis, was <1.0% of the amount of labeled Hx or Ino added. This amount did not change significantly with time, so we subtracted this background value from all measurements to obtain net values for the amount of 3H released enzymatically by IMPDH. The enzyme activity, determined from the slope of the graph of 3H release vs time, was expressed as disintegrations per minute per minute (dpm/min).

Determination of IMPDH activity in whole blood. To determine IMPDH activity in whole blood, we used 300 μL of whole blood instead of lymphocytes in the procedure described above. In some experiments MPA was added to the blood 30 min before analysis.

Effect of leukocyte (WBC) concentration on IMPDH activity. To determine the effect the WBC concentration in whole blood might have on total IMPDH activity, we layered whole blood over an equal amount of Polymorphoprep (Nycomed Pharma, Oslo, Norway). After centrifuging for 30 min at 600g we removed the two layers of WBC (polymorphonuclear and mononuclear cells) and washed them twice with isotonic saline. The erythrocyte layer (devoid of WBC) was also washed twice with saline and then resuspended in plasma; this represented the zero WBC standard. The isolated WBC were also resuspended in patients' plasma and used to reintroduce WBC at cell concentrations of 2.5, 5.0, 10.0, and 25.0 × 10^9/L. The WBC were counted in an automated counter (Coulter STKS; Coulter Diagnostics, Hialeah, FL), after which the IMPDH activity in the samples was determined. The experiments were performed on three separate days (n = 6).

Statistics. Unless specified, all experiments were performed in duplicate on at least three separate occasions. Results are expressed as mean ± SE. Statistical differences between samples were tested by a two-way analysis of variance or paired t-tests, with use of SPSS statistical software (SPSS, Chicago, IL).

Drug Measurement

We quantified RS by HPLC as previously described (21) with some modifications. Briefly, to 0.5 mL of sample we added 1.5 mL of water, 100 μL of a stock solution of internal standard RS-60461-000 (10 000 μg/L), and 0.5 mL of 0.15 mol/L HCl and applied the sample to a pretreated Supelclean C18 (Supelco, Bellefonte, PA) solid-phase extraction column. After washing the column with 1.0 mL of water, we eluted the sample with 1.0 mL of methanol in 0.1 mol/L acetic buffer, pH 4.0 (80:20, by vol). For chromatographic separation of MPA and the internal standard, we used a C18 Novapak HPLC column (Waters, Milford, MA) and a mobile phase of acetonitrile in 5 mL/L phosphoric acid (27:73, by vol). The chromatographic eluate was monitored at 254 nm, and the concentration of MPA was determined by the ratio of its peak height in relation to that of the internal standard. The detection limit of the assay was 400 μg/L, and the linear range extended up to 6000 μg/L (concentrations greater than this were appropriately diluted with drug-free plasma). Between-run coefficients of variation were <10% at 500 μg/L and <7% at 1000 μg/L.

Results

To determine the effect of MPA concentrations on IMPDH activity, we added the drug in concentrations ≤50.0 mg/L to whole blood and to tissue culture medium in which isolated lymphocytes were suspended. The vehicle used, methanol-Tween 80 (70:30 by vol), did not affect IMPDH activity, the final methanol concentration being <2 mL/L. As shown in Fig. 1, the concentration of MPA required for 50% inhibition of IMPDH activity was similar for both whole blood and isolated lymphocytes: 2.0–5.0 mg/L. However, the con-
The concentration of MPAG required for 50% inhibition of enzyme activity was greater than the concentrations tested; at 100 mg/L MPAG, enzyme inhibition was only 74%. Similar results were obtained for rabbit whole blood. Although complete inhibition of IMPDH was not observed at the highest concentration of MPAG investigated, solubilizing the cells in the presence of Triton X-100 (Sigma Diagnostics), 1 mL/L (which alone did not inhibit enzyme activity), completely inhibited enzyme activity at an MPA concentration of 20 mg/L. This suggests that any further metabolism of XMP or excess IMP to uric acid, which would result in the release of $^{3}$H from the C-8 position, is insignificant. The lowest concentration of MPA that produced a significant inhibition of enzyme activity was 1.0 mg/L. In a separate experiment, we measured IMPDH activity in lymphocytes that had been isolated from whole blood containing MPA at 10.0 mg/L. No inhibition of IMPDH activity was observed, which suggests a reversal of MPA-induced inhibition during the isolation of the lymphocytes. For this reason, the rest of the analyses were performed with whole blood as the medium for analysis.

To determine the effect of WBC count on IMPDH activity, we added these cells (0.0–25.0 $\times$ 10$^{9}$/L) to whole blood devoid of WBC and then measured enzyme activity. The results (Fig. 2) indicate a sigmoidal relationship between the two, with the greatest change in activity occurring between WBC concentrations of 2.5 $\times$ 10$^{9}$ to 10.0 $\times$ 10$^{9}$/L. This suggests that for intra- and interpatient comparisons, results should be normalized for WBC concentration. Therefore, all values, unless otherwise specified, were normalized to a WBC concentration of 1.0 $\times$ 10$^{9}$/L.

Table 1 summarizes the intra- and interassay precision of the method for determination of IMPDH activity in whole blood from a single individual, as well as the interindividual variation from blood specimens (both normalized and not normalized for WBC) obtained from several healthy volunteers. The within- and between-run CVs for blood specimens from a single individual were 11% and 16%, respectively. The interindividual variation in IMPDH activity in whole blood (either human or rabbit) was not reduced by correcting the results for WBC content (Table 1). However, this analysis was performed with healthy subjects, who exhibited minimal interindividual variation of WBC count; this is in contrast to transplant patients.

To evaluate the effect of three common anticoagulants (lithium heparin, EDTA, and acid–citrate–dextrose) on IMPDH activity in whole blood, we stored anticoagulated whole-blood samples for as long as 7 days before determining their IMPDH activity. The results indicated no statistical difference ($P > 0.05$) between any of the anticoagulants, and there was no trend to decreased activity for specimens stored at 4°C for up to 4 h; for longer storage times, a trend to decreased activity was noted.

The effect of incubation time with MPA on IMPDH activity in whole blood was determined by adding MPA (1.0 and 10.0 mg/L) to blood and incubating for $\leq$120 min before assaying the enzyme activity. The inhibition of IMPDH by MPA is immediate, and no change in activity was seen during the time studied. To relate the

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**Table 1. Assay precision and interindividual variation of IMPDH activity in whole blood.**

<table>
<thead>
<tr>
<th>Precision</th>
<th>n</th>
<th>Nonnormalized</th>
<th>Normalized$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraassay</td>
<td>6</td>
<td>47.8 ± 5.16</td>
<td>57.5 ± 26.2</td>
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<tr>
<td></td>
<td></td>
<td>(11)$^c$</td>
<td>(16)$^c$</td>
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<tr>
<td>Interassay</td>
<td>7</td>
<td>81.6 ± 13.2</td>
<td>61.6 ± 22.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(16)$^c$</td>
<td>(26.6–105.4)$^d$</td>
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$^a$ All results corrected for background counts.
$^b$ Results normalized for WBC to $1 \times 10^9$/L.
$^c$ CV.
$^d$ Range of values.
concentration of MPA to inhibition of IMPDH in vivo was not possible in patients, so we chose to investigate this in rabbits (the IC50 of MPA for IMPDH in rabbit whole blood being similar to that in human blood). From four rabbits given a single dose of RS, we collected blood specimens at various times after injection, up to 24 h, and measured the MPA concentration and IMPDH activity in each. The results (Fig. 3) indicate an inverse relationship between drug concentrations and IMPDH activity. The peak concentrations of MPA were achieved at 0.25 h after injection and inhibited IMPDH activity by ~60%, similar to the findings for the in vitro studies (~70% inhibition) (Fig. 1). As the MPA concentration decreases throughout the dosing interval, the enzyme activity is gradually restored, returning completely to predose values by 24 h after dosing.

Discussion

Among the approaches used to assess the appropriateness of a dosing regimen for immunosuppressive drugs are the assessment of clinical response and the measurement of drug concentration, relating the drug content to a clinical or toxicological response. A third approach, pharmacodynamics, involves measuring the biological effect of the drug. This latter approach may have significant advantages over the measurement of drug concentrations, especially in multiple drug therapy, where assessment of the appropriate therapeutic range may be difficult. We chose to investigate this approach by using the immunosuppressive drug MPA: Measurement of the biological effect of MPA in lymphocytes is relatively straightforward and the drug will be routinely used in combination with other immunosuppressive agents, thus posing difficulty in establishment of its therapeutic range.

First, we had to establish a reproducible assay for monitoring IMPDH activity in whole blood, a medium that would require minimal sample preparation. The assay is based on the release of 3H from labeled Hx and Ino, substrates of IMPDH (19). IMPDH exists in two isoforms, type I and type II (22). During lymphocyte stimulation, the type II isoform is up-regulated with no change in the type I isoform (22). The development of type II-specific drugs may provide improved selectivity in immunosuppressive therapy. Because MPA inhibits IMPDH noncompetitively with respect to each substrate, IMP and NAD+, the MPA might bind to the enzyme after a tertiary complex is formed: enzyme, IMP, and NAD+ (22). This inhibitory mechanism is the same for both isoforms, but the type II isoform is 3.9-fold more sensitive to MPA than is the type I isoform (22). The selective inhibition of the type II IMPDH may help explain the relatively mild side effects of mycophenolate mofetil observed in phase I clinical trials (13).

We believe that the assay established for IMPDH as described here most probably monitors the activity of both isoforms; however, this needs to be confirmed. The method is relatively simple to perform, and as many as 12 specimens can be processed at the same time. The IC50 for IMPDH was ~2.0–5.0 mg/L for both whole-blood samples and isolated lymphocytes. The detection limit of the assay method for MPA is 1.0 mg/L; at concentrations >20 mg/L, no further inhibition of enzyme activity was observed, suggesting a linearity range of 1.0–20 mg/L. This concentration range spans those concentrations found in plasma after immunosuppressive doses of the drug are administered (13, 14). The IC50 of MPAG, the glucuronide metabolite of MPA, was >100 mg/L, which indicates that its inhibitory effect on IMPDH activity is <5% of that of MPA. This suggests that on a mass basis (mg/mg), the metabolite will contribute minimally to overall immunosuppression. IMPDH activity was not inhibited in lymphocytes isolated from whole blood containing MPA. During the isolation procedure, MPA appears to be lost from cells, thereby resulting in the restoration of enzyme activity; this indicates that the inhibition of IMPDH by MPA is reversible. The inhibition of enzyme activity by MPA is almost immediate when drug is added to whole blood, indicating a rapid uptake of the drug into cells. However, the uptake may be saturable: We did not completely inhibit IMPDH activity even at high concentrations of MPA, except when cell membranes were solubilized in the presence of a nonionic detergent. Therefore, MPA apparently is not readily transported across cellular membranes to achieve the intracellular concentrations that would completely inhibit the IMPDH.

The assay exhibits sufficient analytical precision for its intended use. Although some variation in enzyme activity was noted between patients, this was not substantially reduced when specimens were corrected for WBC. However, this characterization was evaluated in a normal population. Given the greater variation in WBC seen in transplant recipients, we recommend that values be normalized for WBC content because IMPDH activity varies proportionally with the latter. The enzyme activity was not affected by the type of anticoagulant used and was stable in blood specimens stored for as long as 4 h at 4°C. Further investi-
gations are underway to ascertain the effect of storage of specimens for longer periods of time.

To investigate the relationship of the concentration of MPA to inhibition of IMPDH activity in vivo, we chose to use a rabbit model. The IC$_{50}$ of MPA in rabbit whole blood is similar to that obtained in human blood, and we have previously used this model to investigate the pharmacokinetics of RS (14). Measurement of IMPDH activity in whole blood and MPA concentrations in plasma of rabbits administered a single dose of RS indicates an inverse relationship between IMPDH and MPA. Maximal inhibition of IMPDH activity (~60%) occurs at peak concentrations of MPA; as the concentration of the drug decreases postdose, the enzyme activity gradually increases, with little or no inhibition being observed by 24 h. Thus, there may be only a partial inhibition in IMPDH activity throughout the dosing interval, although this may be sufficient to alter the balance of the purine nucleotides within lymphocytes, resulting in impaired synthesis of DNA and of the glycoproteins involved in the adhesion process.

The concept of investigating the biological response to drugs as an alternative to measurement of drug concentrations is being investigated for other drugs, e.g., cyclosporin, which mediates its immunosuppressive activity through the inhibition of calcineurin, a calcium and calmodulin-dependant phosphatase (23–25). With the number of new immunosuppressive drugs recently released (e.g., FK506) or currently undergoing clinical evaluation (e.g., rapamycin, cyclosporin G), establishment of therapeutic ranges for each of the drugs when used in combination with each other will be difficult (26). Measuring the biological response to each specific drug may provide a viable alternative to traditional therapeutic drug monitoring. However, for this to occur, robust assays that can accommodate a large number of specimens within a clinically relevant turnaround time need to be developed and clinically validated. The assay developed for IMPDH described here meets many of the analytical requirements detailed above. Studies investigating the clinical usefulness of this assay in transplant patients chronically administered RS as part of their immunosuppressive drug regimen are now underway.

References