Real-Time PCR Determination of IMPDH1 and IMPDH2 Expression in Blood Cells

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Background: Inosine monophosphate dehydrogenase (IMPDH) catalyzes the rate-limiting step in de novo guanine nucleotide synthesis and is implicated in cell cycle control. Inhibition of this enzyme is associated with immunosuppressive, antiviral, and antitumor activity. IMPDH basal activity increases after initiation of immunosuppressive therapy.

Methods: A real-time reverse-transcription PCR assay was developed and validated for mRNA quantification of the 2 human IMPDH isoforms. Target gene expressions were normalized to the geometric mean of 3 housekeeping genes. Assay utility was tested by analyzing patient samples and cultured cells exposed to immunosuppressive drugs such as the IMPDH inhibitor mycophenolic acid.

Results: The assay was linear over 6 logs of cDNA input and demonstrated specific quantification of IMPDH1 and IMPDH2 expression in cultured cells and patient samples. Limits of detection and quantification were 10 and 10⁴ copies of cDNA per reaction, respectively. Within-run and total between-day CVs were <15% for normalized expression. Changes in IMPDH1 and 2 expression were observed in patient samples after initiation of an immunosuppressive regimen that included calcineurin inhibitors, mycophenolate mofetil, and steroids.

Conclusions: This assay can be used to study the regulation of IMPDH expression and the involvement of the enzymes in immunological and malignant proliferative conditions. This may contribute to the processes of drug development and to the establishment of monitoring strategies for treatment effect and disease activity. © 2007 American Association for Clinical Chemistry

Inosine monophosphate dehydrogenase (IMPDH) is the rate-limiting enzyme in de novo synthesis of guanine nucleotides. Two closely related human IMPDH isoforms, types 1 and 2, have been identified, each consisting of 514 amino acids with 84% sequence identity (1). The isoforms are encoded by 2 distinct genes, IMPDH1 and IMPDH2 (inosine monophosphate dehydrogenase 1 and 2), located at 7q31.3–q32 and 3p21.2–p24.2, respectively (2, 3). Gene expression of the 2 isoforms is differently regulated in various tissues and cell populations, and they are not mutually redundant (4, 5). Inhibitors of IMPDH lead to depletion of guanine nucleotide pools, resulting in various immunosuppressive, antiviral, and antitumor effects. Proliferating lymphocytes are particularly sensitive to IMPDH inhibition owing to their strong dependency on de novo guanine nucleotide synthesis (6). Mycophenolic acid (MPA), a noncompetitive inhibitor of IMPDH, is included in immunosuppressive drug regimens to prevent graft rejection after organ transplantation (7).

Studies in yeast and human cell lines indicate IMPDH gene expression feedback regulation by guanine nucleotides (8, 9). Baseline IMPDH enzyme activity is upregulated in erythrocytes and whole blood after transplantation and initiation of immunosuppressive therapy (10–12). Methods for IMPDH1 and 2 mRNA quantification rely mainly on Northern blotting assays. Real-time reverse transcription (RT)-PCR is the most sensitive method to measure changes in mRNA concentrations. Published real-time RT-PCR assays include a method for IMPDH2.

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Nonstandard abbreviations: IMPDH, inosine monophosphate dehydrogenase; MPA, mycophenolic acid; RT, reverse transcription; MMF, mycophenolate mofetil; RIN, RNA integrity number; Cp, crossing point.

Human genes: IMPDH1 and IMPDH2, inosine monophosphate dehydrogenase 1 and 2; CFLAR, CASP8 and FADD-like apoptosis regulator; ALAS1, 6-aminolevulinate synthase 1; B2M, β2-microglobulin; G6PD, glucose-6-phosphate dehydrogenase; HPRT1, hypoxanthine phosphoribosyltransferase 1; and lMRS, hydroxymethylbilane synthase (formerly PBGD, porphobilinogen deaminase).
quantification (13) and an assay for IMPDH1 and 2 expression in sheep (14). Both assays normalize target gene expression to a single housekeeping gene.

Our goal was to study changes in IMPDH1 and 2 expression in different blood cell populations to obtain further insight into mechanisms underlying these activity changes and provide further information on the contribution of each isoform. We report here an RT-PCR–based assay combining the determination of expression of both human IMPDH isoenzymes based on normalization to a housekeeping gene index.

**Materials and Methods**

**PATIENTS AND SAMPLES**

We collected whole blood specimens from healthy volunteers (n = 8) for assay development. To evaluate the utility of the assay we also obtained specimens from individuals enrolled in 2 ongoing studies focusing on the impact of immunosuppressive drugs on IMPDH expression. One study included renal allograft recipients, from whom samples were drawn predose on 11 occasions before they received transplants and during the 1st 2 weeks posttransplantation. Routine immunosuppression included cyclosporine, mycophenolate mofetil (MMF), and steroids. The 2nd study included healthy volunteers receiving a single dose of MMF (0.25–1.0 g), followed by IMPDH expression measurement in 9 samples drawn predose and the first 24 h after dose. The Regional Committee for Medical Research Ethics approved both studies, and informed consent was obtained from all participants. The assay was also adapted to measure IMPDH expression in cultured lymphoblasts (MOLT-4; European Collection of Animal Cell Cultures).

To assess the expression profile in whole blood cells, we collected 2.5 mL blood in PAXgene Blood RNA tubes (PreAnalytiX). We isolated CD4+ cells from 3 mL EDTA blood using paramagnetic beads coated with CD4 antibodies (Dynal Biotech) according to the manufacturer’s instructions, except for a shortened incubation time of 15 min and 2 washes with PBS. We isolated erythrocytes from 2 mL EDTA blood by filtration through a column containing α-cellulose and microcrystalline cellulose (15). We lysed isolated blood cells with lysis/binding buffer and transferred to sample cartridges. We transduced lysates of erythrocytes, CD4+ cells (4–9 x 10^6), and MOLT-4 cells (2–8 x 10^6) to sample cartridges after thawing and vortex mixing. Total RNA was extracted from 720-μL aliquots and eluted in 50 μL buffer using the MagNA Pure instrument (Roche). RNA was stored at −70°C until further processing.

**RNA analysis.** We measured RNA concentration and purity spectrophotometrically on the NanoDrop ND-1000 instrument (NanoDrop Technologies). We assessed RNA quality by electrophoresis using the RNA 6000 Pico LabChip Kit on the Agilent Bioanalyzer 2100 (both Agilent Technologies). The RNA integrity number (RIN) algorithm (Agilent 2100 expert software) and calculations of the 28S-to-18S rRNA band intensity ratio were used to evaluate RNA integrity.

**REVERSE TRANSCRIPTION**

We used MS2 RNA (Roche) as carrier RNA in all steps at a final concentration of 10 mg/L. The reverse transcription reaction was performed in a thermal cycler (PTC-0150; MJ Research). We compared 2 different reverse transcription enzymes, Transcriptor (Roche) and SuperScript III (Invitrogen), and 2 different priming strategies, random hexamers (Roche and Invitrogen), and oligo(dT) primers (Invitrogen). We tested the reverse transcription linearity by running RT-PCR on serial dilutions of isolated RNA and RNA stock solutions (Roche).

The optimized reverse transcription reaction involved preincubation of a 13-μL mixture of 5 μL total RNA, 0.08 A260 random primers p(dN)10 and H2O (PCR grade) before adding Roche reaction buffer, 20 U Roche RNase inhibitor, Roche dNTP-Mix (1 mmol/L each), and 10 U Transcriptor Reverse Transcriptase to a total volume of 20 μL. Reaction parameters are listed (see Table 1 in the online Data Supplement that accompanies this article at www.clinchem.org/content/vol53/issue6). We stored all cDNA samples at −20°C until analysis.

**QUANTITATIVE REAL-TIME PCR**

**Real-time PCR.** We performed real-time PCR on the Roche LightCycler 1.0 instrument. SYBR Green I (Roche) was used to monitor the amplification during the optimizing stages, and hybridization probes were used for quantification.

We titrated template concentrations to identify potential PCR inhibitors. The optimized reaction mixture contained 2 μL cDNA, 3.5 and 4.5 mmol/L MgCl2 for IMPDH1 and 2, respectively, 0.5 μmol/L sense primer and 1 μmol/L antisense primer for both target genes, 0.225 μmol/L donor and acceptor probe for both target genes, LightCycler Fast Start DNA Master Hybridization Probes reagent, and PCR-grade H2O to a final volume of 20 μL. The reaction parameters are provided in Table 1 of the online Data Supplement.

The crossing point (Cp), defined as the PCR cycle where the fluorescence rises above background noise, was calculated automatically by the 2nd-derivative maximum method (LightCycler Software version 3.5; Roche).
Oligonucleotide design. Sequences for IMPDH1 and 2, 2 known pseudogenes, and CFLAR (CAS8 and FADD-like apoptosis regulator) were obtained from GenBank. Gene-specific primers were designed using Oligo 6.60 software (Molecular Biology Insights); hybridization probe design additionally involved the LightCycler Probe Design software (Roche) combined with manual evaluation of alternative sequences. We also used the software to assess the probability of primer dimer or hairpin formation and to estimate melting temperatures. We checked the primer and probe target sequences for possible homologies with other sequences by applying Basic Local Alignment Search Tool searches. Primers and probes (see Table 2 in the online Data Supplement) were synthesized by TIB Molbiol.

Primers were designed to give amplification products spanning different exons to selectively amplify cDNA and not genomic DNA. All samples were treated with DNase I (Roche) during RNA purification.

Quantification. We evaluated 5 housekeeping genes (housekeeping gene selection set; Roche): δ-aminolevulinate synthase 1 (ALAS1), β₂-microglobulin (B2M), glucose-6-phosphate dehydrogenase (G6PD), hypoxanthine phosphoribosyltransferase 1 (HPRT1), and hydroxymethylbilane synthase (HMBS; formerly PBGD, porphobilinogen deaminase), as potential references for relative quantification. The first 3 genes were selected for normalization. In the CD4⁺ cells, however, only ALAS1 and B2M were selected, because there was a tendency toward coregulation of G6PD and IMPDH1. We assayed all samples and a cDNA calibrator, included in each amplification reaction, in triplicate. Quantification of target gene expression was relative to the geometric mean expression of the selected housekeeping genes in the same sample. This ratio was normalized to the corresponding ratio calculated for the calibrator. Gene-specific amplification efficiency values were used in these calculations to adjust for efficiency differences. An included gene-specific calibrator (10⁶ copies) served as a positive control and allowed estimation of absolute template concentrations by use of preformed calibration curves.

Data Analysis
We analyzed results of the RT-PCR assay with LightCycler Software version 3.5. Relative gene expression calculations, including housekeeping indexes, were performed using LightCycler Relative Quantification software version 1.0 (Roche) and REST-384 software version 1 (http://www.gene-quantification.de). We evaluated gene expression changes using the Wilcoxon signed-rank test (SPSS version 13.0), and defined P values <0.05 as significant.

Assay Validation
Specificity. PCR products were run on 1% agarose gels (ethidium bromide, ultraviolet detection), and melting curve analyses were performed on the LightCycler directly after amplification. We sequenced IMPDH1 and 2 amplification products using BigDye Terminator Chemistry on an ABI Prism 3100 genetic analyzer (Applied Biosystems). Reverse transcriptase negative controls and template negative controls were included in the PCRs.

Calibration curves. We constructed calibration curves to determine reaction efficiencies, linearity, detection and quantification limits, and to relate Cp values to template concentrations. PCR products were purified by agarose gel (1.2%) electrophoresis, excised, and eluted from the gel using a Montage DNA Gel Extraction Device (Millipore). An equimolar quantity of each gene-specific PCR product was serially diluted, and 4–6 replicates of each concentration (10⁶–10 copies/reaction) were amplified. We created calibration curves by plotting the log number of cDNA copies corresponding to each calibrator vs their corresponding Cp value. To assess a possible effect of matrix components on the PCR efficiency, we amplified a dilution series of heterogeneous cDNA samples. The PCR efficiency was calculated from the slope of the calibration curve according to the equation $E = 10^{(-1/slope)}$ (16). We constructed calibration curves on 5 independent occasions and determined the repeatability of the slope and intercept.

Imprecision. We measured assay imprecision by calculating the CV of relative concentrations and normalized ratios of gene expression. We assessed the variability of the reverse transcription step by synthesizing cDNA on 3 days and amplifying these cDNAs in a single PCR run. Imprecision of the post–reverse transcription steps was calculated for the entire dynamic range based on the replicates that generated the calibration curves. Calibrator stability and the imprecision of the post–reverse transcription steps were determined in 20 calibrator samples run repeatedly over a 2-year period. To assess within-run variability of the complete RT-PCR assay, we measured relative IMPDH1 and 2 expression in 5 replicates originating from the same whole blood sample. This was repeated on 3 occasions to measure the total RT-PCR between-run imprecision.

Results
RNA isolation. Isolation of total RNA resulted in concentrations of 7–28, 4–26, and 2–13 mg/L with PAXgene, CD4⁺ cell, and reticulocyte samples, respectively. Yields were donor dependent, probably resulting from fluctuations in the number of blood cells. The mean 260/280 nm absorption ratios were >1.9 for all sample types. The mean RIN values were 8.3, 9.5, and 6.7 for PAXgene, CD4⁺ cell, and reticulocyte samples, respectively. The 28S:18S rRNA band intensity ratio ranged from 0.4 to 1.9. The shapes of the amplification and melting curves were similar for samples and positive controls, indicating the presence of intact RNA.
Reverse transcription. Random hexamer priming was more efficient than oligo(dT) priming for IMPDH1 and the selected housekeeping genes, decreasing Cp by 0.3 to 2.0 cycles. For IMPDH2, oligo(dT) priming decreased Cp values by 0.4 cycles. Transcriptor was preferred over SuperScript III, since it resulted in a slightly greater yield and a shortened incubation time of 15 min. The reverse transcription reaction proved to be linear from $5 \times 10^6$ to $5 \times 10^9$ RNA copies, and a constant volume of total RNA was used in the reverse transcription reaction for further applications.

Amplification. PCR efficiencies for IMPDH1 and 2 were 2.03 and 1.99, whereas the efficiencies for ALAS1, B2M, and G6PD were 1.99, 1.99, and 1.96, respectively. Amplification of a heterogeneous cDNA pool showed PCR efficiencies similar to those of the purified cDNA, and therefore calibration curves for further applications were based on the latter material. Depending on the gene, material, yield, and patient status, the Cp values ranged from 19 to 38 cycles. Reticulocyte and PAXgene samples resulted in the highest ($IMPDH1$ 30–38; $IMPDH2$ 31–36; $n = 30$, day 1) and lowest ($IMPDH1$ 26–30; $IMPDH2$ 26–31; $n = 30$, day 1) Cp values, respectively.

Expression analysis

All tested housekeeping genes were detectable in the relevant samples. HPRT1 was excluded because of its role in purine metabolism, suggesting coregulation with IMPDH (4). HMB5 expression was lower compared with the target genes (in a representative series, $n = 4$ samples, HMB5 Cp ranged from 36 to 39, compared with $IMPDH1$ 30–32 and $IMPDH2$ 30–33). Expression and reaction kinetics were similar for the selected housekeeping genes, ALAS1, B2M, and G6PD, and the target genes. Expression results obtained from CD4$^+$ cell samples indicated potential coregulation of G6PD and IMPDH1. With the exception of G6PD in CD4$^+$ cells, results obtained after separate normalization against each of the housekeeping genes showed the same trends as the results after normalization to the geometric mean of the expression. The target and reference gene expression results showed the following tendencies in the tested sample types: $B2M > G6PD > IMPDH1 \approx IMPDH2 > ALAS1$. The differences between IMPDH1 and 2 expressions were largest in the CD4$^+$ cells.

Assay validation

PCR product sequencing demonstrated specific amplification of IMPDH1 and 2. The melting temperatures were 90.1 and 86.3 °C for the IMPDH1 and 2 amplicons, and no contaminating products or primer dimers were observed. Gel electrophoresis resulted in single bands corresponding to the expected length of $\sim 190$ bp.

Calibration was linear over a dynamic range of at least 6 orders of magnitude, corresponding to starting concentrations of $10^6$–10 templates per reaction; Pearson correlation coefficients were $-0.99$ for both IMPDH1 and 2 (see Fig. 1 in the online Data Supplement). Repeated generation of the calibration curves resulted in CVs $<1.7\%$ and $<0.8\%$ for slope and intercept, respectively. Assay sensitivity was confirmed by the slopes (absolute values 3.04–3.26) of the linear regression curves used to measure the dynamic range (see Fig. 1 in the online Data Supplement). The detection limit was 10 templates per reaction, defined as the lowest starting template concentration resulting in an amplification curve. The lower limit of quantification was set to $10^3$ templates per reaction, since lower concentrations resulted in a significant increase in imprecision, from CV $\approx 12.0\%$ to $\approx 25.7\%$ (Fig. 1).

The total between-day RT-PCR CVs were 10.4% and 13.4% (normalized values) for IMPDH1 and 2, respectively. Further details of assay imprecision are given in Table 1 and Fig. 1. The between-day CVs specific for the

| Table 1. Imprecision in reference normalized target gene expression. |
|-------------------|-------------------|-------------------|
|                   | IMPDH1, CV %      | IMPDH2, CV %      |
| Calibrator-normalized | 6.4               | 12.8              |
|                    | Within-run, n = 5  | Total, n = 15     |
|                    | 10.4              | 13.4              |
| Not calibrator-normalized | Total, n = 20     | 10.6              |
|                   |                   | 14.0              |
| a Five replicates were analyzed on 3 separate days. |
| b Determined over a 2-year period and based on the relative gene expression in the calibrator. |
reverse transcription reaction were 2.0% and 5.5% for IMPDH1 and 2, respectively.

APPLICATION
We applied the assay to a limited number of samples from a renal transplant recipient. Initiation of immunosuppressive therapy after transplantation was associated with fluctuations in IMPDH1 and 2 expression (Fig. 2). Within the 1st 1–4 h after a single dose of MMF, healthy volunteers demonstrated downregulation of IMPDH1 and upregulation of IMPDH2. Samples drawn on 4 occasions from a control individual not receiving immunosuppressive drugs demonstrated no significant changes of IMPDH1 or 2 expression (relative expression range, n = 4; IMPDH1 PAXgene 0.98–1.06, CD4+ cells 0.63–0.69; IMPDH2 PAXgene 0.36–0.41, CD4+ cells 1.94–2.18; i.e., all ranges well within the CV of the assay). Cultured cells incubated with 0.05–0.5 μmol/L MPA for 72 h showed an increase in IMPDH enzyme activity, measured by HPLC, which was reflected by similar changes in IMPDH1 and 2 expression. The maximum increase of ~60% in IMPDH basal activity (from a mean of 39 pmol XMP/10⁶ cells/min), as well as IMPDH2 expression (increase by median factor of 2.03, range 2.0–2.4, n = 3 experiments), was observed after incubation with 0.15 μmol/L MPA.

Discussion
We developed and validated an assay for IMPDH1 and 2 mRNA quantification in whole blood, CD4+ cells, and reticulocytes. The focus of our studies was the impact of the IMPDH inhibitor MPA, and concomitantly administered drugs such as steroids, on the expression of the IMPDH isoforms. We analyzed whole blood cells to study IMPDH1 and 2 expression in relation to the IMPDH enzyme activity measured in whole blood. Lymphocytes, and CD4+ cells in particular, play a pivotal role in graft rejection, and MPA exerts its effects primarily by affecting lymphocytes. We therefore specifically studied CD4+ cells. Erythrocytes constitute the largest population of blood cells and probably influence the whole blood IMPDH enzyme activity. Expression in reticulocytes, which contain some preformed mRNA, could provide a sensitive indicator for changes in the IMPDH expression and activity of erythrocytes, since reticulocytes represent the most newly formed population.

The results with this assay revealed IMPDH1 and 2 gene regulation after initiation of immunosuppressive therapy. This regulation was observed in patient samples as well as in cultured cells, and both steroids and MPA seem to be involved (Fig. 2). In single-dose studies of healthy volunteers, the preliminary data indicate a qualitatively different response. Although these data so far are sparse, they could support the hypothesis that steroids (not administered to the healthy volunteers) may be the most important factor regulating IMPDH expression early after transplantation. The IMPDH expression changes might influence immunosuppressive response and the risk of rejection after transplantation. We will follow up on these findings in the continuation of the mentioned studies.
PAXgene tubes offer a convenient and efficient way to stabilize RNA (17). RNA from CD4+ cells, reticulocytes, and MOLT-4 cells was stabilized in guanidine thiocyanate buffer within an hour after sampling. The RIN values showed isolation of intact RNA from PAXgene and CD4+ cell samples. Reticulocyte samples resulted in lower but acceptable RIN values, probably because of lower RNA content and longer processing time before RNA stabilization. The 28S:18S ribosomal RNA band ratio was dependent on RNA input concentration and showed a larger degree of variability. This assay includes gene sequences shorter than 200 nucleotides, which usually can be successfully reverse transcribed and amplified even when RNA is partially degraded.

This is the first RT-PCR assay using hybridization probes for specific quantification of both human IMPDH1 and 2. Variations in cell numbers and populations occurring because of drug treatment or immunological conditions might contribute to the observed variation in yield and expression. Single-template detection is theoretically feasible in real-time RT-PCR, but distribution statistics at low template concentrations influence the actual lower limit of detection, as illustrated by the increase in CVs with decreasing template concentrations shown in Fig. 1. Low-template reactions may also lead to underestimation of the copy number due to relatively higher unspecific background amplification competing for reagents. The log-linear inverse relationship between concentrations and Cp values indicates that CVs based on relative concentrations will be nominally higher than CVs based on Cp values. We calculated CVs in terms of relative concentrations and the final normalized ratios. These CVs are directly applicable and relevant for the interpretation of results presented in terms of relative concentrations. Within-run and total imprecision were <25%, which may be an acceptable limit in this type of assay. Repeated analysis of the calibrator sample, already included in each run, demonstrated the stability of the cDNA material over a 2-year period (Table 1).

We determined gene-specific PCR efficiencies and used them in the expression calculations to correct for gene- and assay-specific factors. The linear calibration curves indicate constant amplification efficiencies over the concentration range studied, confirming minimal influence of PCR inhibitors.

Relative expression results may be influenced by interindividual differences in housekeeping gene expression. When fluctuations over time are studied in individuals, such variability will be of less importance. Normalization of the target gene expression to an expression ratio of several housekeeping genes further compensates for this, and for variations in the sample amount, RNA recovery, RNA integrity, efficiency of cDNA synthesis, presence of inhibitors, and differences in the overall transcriptional activity of the tissues or cells analyzed. Earlier published methods use normalization to a single housekeeping gene such as glyceraldehyde-3-phosphate dehydrogenase or β-actin (13, 14). There is, however, no known ubiquitous, stably expressed reference gene. To strengthen the accuracy of this assay, the target gene expressions were normalized to the geometric mean expression of 3 reference genes, a housekeeping gene index. Reference genes were selected among ubiquitously expressed genes from different functional classes to reduce the influence of coregulation.

**CLINICAL APPLICATIONS**

The association between increased IMPDH activity and cellular proliferation and malignant transformation makes this enzyme a potential target for treatment in organ transplantation and for malignant disorders. The 2 IMPDH isoforms are associated with different functions and gene regulation, and proliferation and neoplastic transformation are reported to be linked to increased IMPDH2 expression (18, 19). Assessment of the relative importance and contribution of the IMPDH isoforms to intracellular processes in various disease states will provide information to establish whether both enzymes are valid therapeutic targets. Elucidation of the association between the IMPDH isoforms’ gene expression and immunosuppression or malignancy might also provide an opportunity for the monitoring of treatment effect or disease activity. The presented IMPDH1 and 2 expression assay will be a useful tool for such applications.

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**References**