Pitfalls in the Diagnosis of Patients with a Partial Dihydropyrimidine Dehydrogenase Deficiency

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Background: Dihydropyrimidine dehydrogenase (DPD) catalyzes the degradation of thymine, uracil, and the chemotherapeutic drug 5-fluorouracil. To identify patients suffering from complete or partial DPD deficiency and to identify pitfalls that can preclude the proper diagnosis of patients with partial DPD deficiency, a sensitive and accurate assay is necessary.

Methods: The activity of DPD was measured using [4-14C]thymine followed by separation of substrate and products with reversed-phase HPLC with on-line detection of the radioactivity.

Results: Complete baseline separation of radiolabeled thymine and all degradation products was achieved within 15 min. The detection limit for dihydrothymine was 0.4 pmol. In lymphocytes, the DPD activity deviated from linearity at low protein concentrations (<0.2 g/L). Profoundly decreased activity of DPD was detected in the peripheral blood mononuclear cells (PBM cells) of two tumor patients when measured at low protein concentrations. Low DPD activity comparable to that observed in obligate heterozygotes was initially detected in PBM cells, containing substantial amounts of myeloid cells, from a patient suffering from 5-fluorouracil toxicity. However, after the patient experienced full clinical recovery, normal DPD activity was observed in the PBM cells. No significant differences in DPD activity were observed between exponentially growing fibroblasts and those at confluence. The range of DPD activities of obligate heterozygotes overlaps the range of DPD activities of controls.

Conclusions: The low activity of DPD measured in PBM cells containing myeloid cells or that measured at a low protein concentration in the assay mixture is not indicative of heterozygosity for a mutant DPD allele. Although fibroblasts are suitable to establish a complete deficiency of DPD, unambiguous detection of heterozygotes is not possible.

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In mammalian liver, the pathway for the catabolism of thymine and uracil consists of three consecutive steps (Fig. 1). Dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2) is the initial and rate-limiting enzyme in the catabolism of the pyrimidine bases, and it catalyzes the reduction of thymine and uracil to 5,6-dihydrothymine and 5,6-dihydouracil, respectively. The second step consists of a hydrolytic ring opening, which is catalyzed by dihydropyrimidinase (EC 3.5.2.2). Finally, β-ureidoisobutyric acid (N-carbamyl-β-aminoisobutyric acid) or β-ureidopropionic acid (N-carbamyl-β-alanine) are converted to β-aminoisobutyric acid or β-alanine, ammonia, and CO₂ by β-ureidopropionase (EC 3.5.1.6). In children with a DPD deficiency, a large phenotypic variability was observed, with convulsive disorders, motor retardation, and mental retardation being the most abundant manifestations (1, 2). In these patients, substantial accumulation of thymine and uracil has been detected in urine, blood, and cerebrospinal fluid (2–5).

DPD is also responsible for the breakdown of the widely used antineoplastic agent 5-fluorouracil (5FU), thereby limiting the efficacy of the therapy. 5FU is one of the few drugs that shows some antitumor activity against various otherwise untreatable tumors, including carcinomas of the gastrointestinal tract, breast, ovary, and skin. However, >80% of the administered 5FU is catabolized by DPD (6), and a correlation has been observed between the pretreatment activity of DPD in peripheral blood mononuclear cells (PBM cells) and the systemic clearance of 5FU in cancer patients (7, 8). In line with this phenom-
enon is the recent finding of a pharmacogenetic disorder involving cancer patients with a complete or partial deficiency of DPD and suffering from severe or life-threatening toxicity after the administration of 5FU. It has been shown that three such patients were genotypically heterozygous for a mutant allele of the gene encoding DPD (9–12). Based on population analysis of the DPD activity, the frequency of heterozygotes in the general population has been estimated to be as high as 3% (8, 13). Such patients might be at risk of developing severe toxicity after the administration of 5FU. Considering the frequent use of 5FU in the treatment of cancer patients and the severe 5FU-related toxicity in patients with low DPD activity, the analysis of the DPD activity before the start of treatment with 5FU has been advocated (10, 14, 15).

The activity of DPD can be detected in a variety of tissues, with the highest activity found in liver and monocytes (16, 17). To investigate whether patients suffer from a complete or partial DPD deficiency, PBM cells and fibroblasts are often used. However, a large range of DPD activity has been detected when these types of cells are used, thus hampering proper diagnosis. In this report, we describe a sensitive assay to measure the activity of DPD in a variety of tissues and describe several pitfalls that can preclude the proper diagnosis of patients with a partial DPD deficiency.

**Materials and Methods**

**CHEMICALS**

[4-14C]Thymine (1.85–2.22 GBq/mmol) was obtained from Moravek Biochemicals. LymphoprepTM (specific gravity, 1.077 kg/L, or 280 mOsm) was obtained from Nycomed Pharma AS. LeucoSep tubes were supplied by Greiner. Fetal calf serum was obtained from BioWhit-taker. HAM-F10 medium with 20 mmol/L HEPES was obtained from Life Technologies. CompleteTM mini EDTA free tablets (protease inhibitor cocktail) were obtained from Boehringer Mannheim GmbH Biochemica. All other chemicals were of analytical grade.

**ISOLATION OF HUMAN PBM CELLS**

PBM cells were isolated from 15 mL of EDTA-anticoagulated blood. After centrifugation (250g for 10 min at room temperature) the platelet-enriched plasma sample was removed. The remaining cell pellet was resuspended in “supplemented phosphate-buffered saline” (supplemented PBS; 9.2 mmol/L Na2HPO4, 1.3 mmol/L NaH2PO4, 140 mmol/L NaCl, 2 g/kg bovine serum albumin, 13 mmol/L sodium citrate, 5 mmol/L glucose, pH 7.4) to a final volume of 15 mL. The cell suspension (≥ 7 mL) was layered on top of 3 mL of Lymphoprep (specific gravity, 1.077 kg/L, or 280 mOsm) in 10-mL LeucoSep tubes and centrifuged at 800g at room temperature for 20 min. The interface containing the PBM cells was collected, diluted with supplemented PBS to a final volume of ~15 mL, and centrifuged at 800g for 8 min. To lyse the erythrocytes, the pellet was resuspended in 5 mL of ice-cold NH4Cl solution (155 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L EDTA) and kept on ice for 5 min. After the addition of 10 mL of ice-cold supplemented PBS, the solution was centrifuged at 250g at 4 °C for 10 min. The pellet was washed once more with supplemented PBS, and the cell pellet was resuspended in ~2.5 mL of PBS (9.2 mmol/L Na2HPO4, 1.3 mmol/L NaH2PO4, 140 mmol/L NaCl, pH 7.4). An aliquot was used for cell counting, and the purity of the PBM cells was assessed by morphologic examination of the cell suspension on a cytospin preparation stained with Jenner-Giemsa. The remaining suspension was centrifuged at 11 000g for 10 s. The supernatant was discarded, and the pellet was frozen in liquid nitrogen and stored at −80 °C until further analysis.

**PURIFICATION OF BLOOD CELLS**

Lymphocytes, monocytes, and granulocytes were purified from buffy coats (leukocyte-enriched fractions) from healthy volunteers by centrifugation over Percoll followed by elutriation centrifugation, as described previously (17). The remaining erythrocytes were lysed with isotonic NH4Cl solution at 4 °C. The solution was centrifuged at 400g for 5 min at 4 °C, and the resulting pellet was washed twice with PBS. The final cell preparations were ~99% pure.

**CULTURE CONDITIONS OF HUMAN FIBROBLASTS**

Fibroblasts were cultured from skin biopsies obtained from controls (healthy volunteers and patients admitted to our hospital with clinical and biochemical findings not indicative of inborn errors in the purine and pyrimidine metabolism) and obligate heterozygotes for a DPD deficiency (e.g., parents of a completely DPD-deficient individual).

Biopsies were incubated at 37 °C in HAM-F10 medium supplemented with 20 mmol/L HEPES and 150 mmol/L fetal calf serum in 25-cm2 cell-culture flasks until an adequate number of proliferating cells was obtained. Subsequently, the cells were harvested with 2.5 g/L trypsin and distributed over two 75-cm2 flasks. These cells
were cultured in HAM-F10 medium supplemented with 20 mmol/L HEPES and 100 mL/L fetal calf serum. In one flask, monolayers of fibroblasts were grown to confluence and kept at this stage for 5 additional days. In the other flask, fibroblasts were harvested during exponential growth. Fibroblasts were harvested with 2.5 g/L trypsin, and after the cells were washed once with PBS and twice with 9 g/L NaCl, they were collected by centrifugation (175g at 7 °C for 5 min), and the supernatant was discarded. The pellets were stored at −80 °C.

PREPARATION OF TISSUE HOMOGENATES
The frozen cell pellets of fibroblasts and leukocytes were suspended in 300 μL of an ice-cold solution of 35 mmol/L potassium phosphate (pH 7.4) and 2.5 mmol/L MgCl2. Homogenates (200 g/L) of frozen human livers were prepared in a buffer containing 35 mmol/L potassium phosphate (pH 7.4), 2.5 mmol/L MgCl2, and Complete (1 tablet/10 mL) with the aid of a Teflon glass homogenizer. All homogenates were sonicated three times at 4 W (Vibra-cell Sonificator; output control, 20%) for 10 s with intervals of 30 s under constant cooling in ice-water. After centrifugation (11 000 g at 4 °C for 20 min), aliquots of the supernatants were used for determination of the protein concentrations. Dithiothreitol was added to the remaining supernatants to a final concentration of 1 mmol/L, and the supernatants were subsequently stored in liquid nitrogen until further analysis. Protein concentrations in the supernatants were determined by the copper-reduction method using bicinchoninic acid, essentially as described by Smith et al. (18).

DETERMINATION OF THE DPD ACTIVITY
The DPD activity of fibroblasts and leukocytes was determined in a reaction mixture containing 35 mmol/L potassium phosphate (pH 7.4), 2.5 mmol/L MgCl2, 1 mmol/L dithiothreitol, 250 μmol/L NADPH, and 25 μmol/L [4-14C]thymine. The DPD activity of human liver was determined in a reaction mixture containing 35 mmol/L potassium phosphate (pH 7.4), 2.5 mmol/L MgCl2, 1 mmol/L dithiothreitol, 25 mmol/L NADPH, and 50 μmol/L [4-14C]thymine. The reaction mixture and the sample were equilibrated separately at 37 °C in a stirring water bath for 2 min, and the reaction was started by addition of the sample, with a final volume of 100 μL. After an appropriate incubation time, the reaction catalyzed by DPD was terminated by the injection of 25 μL of 100 mL/L perchloric acid into the reaction tube. The reaction mixture was centrifuged in a microcentrifuge (11 000g for 5 min) to remove the protein. An aliquot of supernatant (5 μL) was mixed with 4 mL of scintillation liquid, and the radioactivity was quantified by scintillation counting. The remaining supernatant was stored at −20 °C and saved for further analysis by HPLC. A blank value was always obtained when water was used instead of a sample.

HPLC ANALYSIS
The separation of radiolabeled thymine from the reaction products 5,6-dihydrothymine, N-carbamyl-β-aminoisobutyric acid, and β-aminoisobutyric acid was accomplished by HPLC after the injection of 70 μL of a supernatant into the HPLC system. The HPLC system consisted of two Waters 510 HPLC pumps, a Waters 680 automated gradient controller (Waters Associates), and a Gilson 231 XL sample injector with a Gilson 401 diluter (Gilson Medical Electronics). The radioactivity was detected on-line with a Radiomatic 525 TR Flow Scintillation Analyzer with a 500-μL TR-LSC cell (Packard Instrument). Scintillation fluid (Ultima Flo AP; Packard) was used at a 1:1 ratio of effluent to scintillation fluid.

HPLC was performed on a reversed-phase column (Alltima C18, 250 × 4.6 mm, 5 μm particle size; Alltech Associates) and a guard column (Supelguard LC-18-S, 5 μm particle size, 20 × 4.6 mm; Supelco). The solvents used for chromatography consisted of 50 mmol/L NaH2PO4, pH 4.5 (solvent A), and a 1:1 mixture (by volume) of 50 mmol/L NaH2PO4, pH 4.5, and acetonitrile (solvent B). The elution of radiolabeled thymine and the reaction products 5,6-dihydrothymine, N-carbamyl-β-aminoisobutyric acid, and β-aminoisobutyric acid was performed isocratically with 100% solvent A at a flow rate of 2 mL/min. Solvent B at a final concentration of 50% was used for the removal of more hydrophobic impurities that were present in the [14C]thymine stock solution. The integration of the data was performed with a FLO-ONE integration package (Packard).

CALCULATION OF THE DPD ACTIVITY
The activity of DPD was calculated according to the following equation:

$$\text{DPD activity (nmol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}) = \left(\frac{\text{cpm}_{\text{b}} - \text{cpm}_{\text{m}}}{\text{eff}} \times \text{inc. vol.} / \text{inj. vol.}\right) \times \text{s.a.}^{-1} \times \text{mg}^{-1} \cdot \text{h}^{-1}$$

where \text{inc. vol.} is the volume of reaction mixture (100 μL) + the volume of perchloric acid (25 μL); \text{inj. vol.} is the injection volume of the HPLC (usually 70 μL); \text{cpm}_{\text{b}} is the counts per minute of the peaks of the various reaction products produced by DPD in the sample; \text{cpm}_{\text{m}} is the counts per minute of the peaks at the position of the reaction products in a blank; \text{Eff.} is the counting efficiency of the Radiomatic \((\text{LSC}_{\text{m}} - \text{LSC}_{\text{b}})/\text{cpm}_{\text{tot}} \times \text{inj. vol.} / \text{counting vol.})\); \text{cpm}_{\text{tot}} is the total counts per minute in the perchloric acid effluent to scintillation fluid. The radioactivity was detected on-line with a Radiomatic 525 TR Flow Scintillation Analyzer with a 500-μL TR-LSC cell (Packard Instrument). Scintillation fluid (Ultima Flo AP; Packard) was used at a 1:1 ratio of effluent to scintillation fluid.
extract determined by the Radiomatic; s.a. is the specific activity of \([4-^{14}C]\)thymine (dpm/nmol); \(mg\) is the amount of protein in the reaction mixture in mg; and \(t\) is the reaction time in hours.

ASSAY VALIDATION

The intraassay variation of the total procedure was assessed by determination of the DPD activity and protein concentration in five replicates of a human lymphocyte sample on the same day. The interassay (between-day) variation was determined by analyzing a human lymphocyte sample on 9 different days. The reproducibility of the assay is expressed as the CV. The detection limit of the HPLC procedure is defined as the baseline noise plus 3 SD.

STATISTICAL ANALYSIS

The correlation between the activity of DPD and the percentage of monocytes or lymphocytes was studied by determination of the Pearson correlation coefficients and linear regression. The differences between the DPD activities of exponentially growing and stationary fibroblasts were analyzed with the paired \(t\)-test. The difference between the mean activities of DPD of control fibroblasts and those of obligate heterozygotes were analyzed with the two-sample \(t\)-test, using the unequal variance method. Significance was set a priori at \(P \leq 0.05\). Analyses were performed with the Statistical Package for the Social Sciences (SPSS).

HPLC PROCEDURE

The DPD activity was measured using radiolabeled thymine followed by separation of substrate and products with reversed-phase HPLC with on-line detection of the radioactivity. Analysis of a particular stock solution of \([4-^{14}C]\)thymine by HPLC showed that the radiolabeled thymine was essentially pure (>99%). The small amount of impurities present in the stock solution of radiolabeled thymine eluted partly in the dead volume of the separation and after the column was cleaned with 250 mL/L acetonitrile, which was performed routinely after each HPLC run. To obtain a highly sensitive assay, the \([4-^{14}C]\)thymine stock was routinely purified by reversed-phase HPLC (Fig. 2A). A complete baseline separation was achieved within 15 min for \(\beta\)-aminoisobutyric acid, N-carbamyl-\(\beta\)-aminoisobutyric acid, 5,6-dihydrothymine, and thymine, with retention times of 1.8, 5.3, 11.4, and 13 min, respectively. After incubation of a homogenate of human lymphocytes with radiolabeled thymine, only 5,6-dihydrothymine could be detected, demonstrating the absence of detectable dihydropyrimidinase and \(\beta\)-ureido-propionase activity (Fig. 2B). In contrast, all three degradation products were detected when human liver homogenates were used, with \(\beta\)-aminoisobutyric acid being the most abundant (Fig. 2C). In the presence of \([2-^{14}C]\)thymine, \(^{14}\)CO\(_2\) was produced by liver homogenates instead of radiolabeled \(\beta\)-aminoisobutyric acid (results not shown). The detection limit, defined as the baseline noise plus 3 SD, of radiolabeled dihydrothymine was \(\sim 0.4\) pmol. The detection limit for the combined analysis of \(\beta\)-aminoisobutyric acid, N-carbamyl-\(\beta\)-aminoisobutyric acid and 5,6-dihydrothymine by HPLC was 2 pmol.
REACTION CONDITIONS
The DPD activity in human liver was linear for protein concentrations of 0.06–2.4 g/L, although at higher protein concentrations a decrease in specific activity was observed. With respect to the time dependence of the DPD activity, linear reaction rates were observed up to 4 h when measured at low protein concentration (0.06 g/L) as well as at high protein concentration (1.2 g/L). In the latter case, the substrate concentration of radiolabeled thymine was increased to 100 μmol/L. In fibroblasts, the reaction catalyzed by DPD was linear for protein concentrations of 0.065–0.65 g/L, whereas a decrease in specific activity was observed at higher protein concentrations. With respect to the time dependence of the reaction catalyzed by DPD, a linear increase in product formation was observed up to 4 h.

In lymphocytes, the reaction catalyzed by DPD was linear with time up to 5 h. Fig. 3A shows that the amount of dihydrothymine produced by DPD from lymphocytes increases linearly with respect to the protein concentration in the range of 0.2–6 g/L. However, at low protein concentrations (<0.2 g/L), a deviation from linearity occurred that could not be prevented by the addition of human serum albumin. This phenomenon is clearly illustrated when the specific activity of DPD is plotted against the protein concentration (Fig. 3B).

ASSAY VALIDATION
The intraassay CV for the determination of DPD activity was 5% (mean ± SD, 4.7 ± 0.2 nmol·mg⁻¹·h⁻¹; n = 5). The interassay CV for the determination of DPD activity was 9% (4.4 ± 0.4 nmol·mg⁻¹·h⁻¹; n = 9).

PBM CELLS
The effect of high and low protein concentrations on the DPD activity in PBM cells of two cancer patients is shown in Fig. 4. In both patients, PBM cells were collected on different occasions during their treatment with fluoropyrimidines. In patient 1, normal DPD activity was detected (9.4 ± 1.4 nmol·mg⁻¹·h⁻¹; n = 9) for protein concentrations between 0.21 and 0.51 g/L (0.31 ± 0.08 g/L). However, a significantly decreased activity of DPD (5.0 ± 0.85 nmol·mg⁻¹·h⁻¹; n = 3) was detected when the DPD activity was measured at a low protein concentration (0.041 ± 0.014 g/L; range, 0.028–0.062 g/L). Similarly, in patient 2, normal DPD activity was detected (9.9 ± 0.95 nmol·mg⁻¹·h⁻¹; n = 8) for a protein concentration of 0.31 ± 0.01 g/L, whereas strongly decreased DPD activity (2.3 nmol·mg⁻¹·h⁻¹) was detected when measured at a protein concentration of 0.04 g/L. For these reasons, we recommend performing the DPD assay in PBM cells using protein concentrations between 0.2 and 1 g/L. In general, 3 × 10⁶ PBM cells are sufficient to perform the DPD activity measurements.

Previously, we demonstrated that the activity of DPD can be detected in all blood cells except red blood cells, with the highest activity of DPD being present in monocytes followed by that of lymphocytes (17). Fig. 5 shows

Fig. 3. Protein dependence of the DPD activity in human lymphocytes (A) and relationship between the specific activity of DPD and the protein concentration (B).

Fig. 4. Relationship between the DPD activity in PBM cells and the protein concentration.

The DPD activity was determined in PBM cells of two cancer patients at high and low protein concentrations. Bars, SD.
that a positive correlation exists between the DPD activity of PBM cells obtained from healthy volunteers and the percentage of monocytes (Fig. 5A). Conversely, a negative correlation was observed between the DPD activity in PBM cells and the percentage of lymphocytes (Fig. 5B).

The DPD activity (3.2 nmol·mg⁻¹·h⁻¹) in PBM cells from patient 1, who experienced severe toxicity after treatment with fluoropyrimidines, was decreased compared with that observed in healthy individuals and comparable to that of obligate heterozygotes (4.3 ± 1.5 nmol·mg⁻¹·h⁻¹; n = 4; Fig. 6). However, morphologic examination of the isolated cells on a cytospin preparation showed the presence of large amounts of granulocytes (37% metamyelocytes, 26% band neutrophils, 17% neutrophils, 15% lymphocytes, and 5% monocytes). Because the DPD activity in granulocytes is twofold lower compared with that observed in lymphocytes, the low DPD activity of patient 1 most probably reflects the presence of large amounts of granulocytes in the isolated cell fraction. The aberrant composition of the isolated PBM cell fraction of this patient might have been caused by the granulocyte colony-stimulating factor that the patient had been receiving as treatment for grade 4 granulopenia. Analogously, low DPD activity (4.2 nmol·mg⁻¹·h⁻¹) was initially detected in PBM cells from patient 2 when she was suffering from grade 4 toxicity after treatment with 5FU. Morphologic examination of the isolated cells obtained during the crisis showed the presence of large amounts of granulocytes (3% metamyelocytes, 64% band neutrophils, 19% neutrophils, and 14% lymphocytes). However, after the patient had achieved full clinical recovery, normal DPD activity (8.6 nmol·mg⁻¹·h⁻¹) was detected in her PBM cells.

**Fibroblasts**

Previously, we observed that the DPD activity in fibroblasts was highly variable between individuals (10). To investigate whether the large range of DPD activities in
The DPD activity was determined in fibroblasts of controls and obligate heterozygotes during exponential growth (Exp.) and after reaching confluence (Confl.). No significant differences were observed between the DPD activity of exponentially growing cells (0.9 ± 0.5 nmol·mg⁻¹·h⁻¹) and stationary cells (1.1 ± 1.2 nmol·mg⁻¹·h⁻¹) of healthy volunteers. In addition, a comparable DPD activity was also present in exponentially growing fibroblasts (0.5 ± 0.4 nmol·mg⁻¹·h⁻¹) and stationary cells (0.4 ± 0.2 nmol·mg⁻¹·h⁻¹) of obligate heterozygotes. The mean DPD activity of exponentially growing fibroblasts of controls was significantly higher (P < 0.05) when compared with exponentially growing cells of obligate heterozygotes. Similarly, a significant difference was noted between the DPD activity of control fibroblasts at confluence and those of obligate heterozygotes (P < 0.05).

Discussion

In this study, we developed a sensitive assay to measure the activity of DPD that is based on the separation of [4-¹⁴C]thymine from radiolabeled 5,6-dihydrothymine, N-carbamyl-β-aminoisobutyric acid, and β-aminoisobutyric acid by reversed-phase HPLC with on-line detection of radioactivity. A complete baseline separation was achieved between thymine and dihydrothymine, using a single column with a detection limit of radiolabeled dihydrothymine of 0.4 pMol. Therefore, detection limit of our assay is 10-fold lower than assays that use radiolabeled 5FU as a substrate for DPD (19, 20). Such a low detection limit is of paramount importance to identify truly DPD-deficient patients because the presence of a small but significant residual activity might be sufficient to synthesize the neurotransmitter β-alanine from uracil and to prevent the occurrence of increased concentrations of uracil and thymine (21). In addition, our analysis time is considerably shorter compared with other HPLC procedures that have been used to separate dihydrofluorouracil from 5FU, thus saving costs and time. These latter procedures require the use of two reversed-phase columns connected in series to obtain adequate separation of 5FU and its metabolites (19, 20). [4-¹⁴C]Thymine was catalyzed by human liver to radiolabeled dihydrothymine, N-carbamyl-β-aminoisobutyric acid, and β-aminoisobutyric acid, showing the presence of all three active enzymes—DPD, dihydropyrimidinase, and β-ureidopropionase—of the catabolic pathway of the pyrimidine bases.

5FU remains a major drug in the treatment of advanced colorectal cancer, and it has been shown that the continuous intravenous infusion of 5FU is superior to bolus injections of 5FU with respect to response rate, whereas only a small increase in median survival was observed (22). In addition, a relationship between the 5FU dose intensity and the therapeutic response has been noted (23, 24). Because 5FU has a relatively narrow therapeutic index, toxicity increases as the dose is escalated (24). The major types of toxicity encountered in patients treated with 5FU are gastrointestinal toxicity, hematological toxicity (mainly neutropenia), and hand-foot syndrome (25). Considering the pivotal role of DPD in the metabolism of 5FU and the fact that patients with low DPD activity suffer from very severe 5FU-related toxicity, it is important to identify those patients who are at risk. In that respect, we and others have shown that detection of a deficiency of DPD can be performed by determination of the DPD activity in PBM cells (9, 10, 26, 27). Recently, it has been shown that patients with advanced squamous cell carcinomas of the head and neck had a changed leukocyte composition with a relative increase in the number of monocytes (28). Furthermore, a major shift was observed among the mononuclear cells to the monocytic lineage during treatment of the patients with radiotherapy or chemotherapy (28). Because our results showed that there is a significant correlation between the activity of DPD in PBM cells and the percentage of monocytes, a differential count of the isolated PBM cells by morphologic examination should always be performed.

The analysis of DPD activity in the leukocytes of two cancer patient demonstrated that the DPD activity was comparable to that observed in obligate heterozygotes. Furthermore, the residual DPD activities in the leukocytes of these cancer patients were comparable to those observed in other patients suffering from 5FU toxicity (9, 10, 27). However, morphologic examination of the isolated cells showed the presence of substantial amounts of contaminating granulocytes. Because the DPD activity in granulocytes is twofold lower than that observed in lymphocytes (17), the low DPD activities in these two patients most probably reflect the presence of large amounts of granulocytes in the isolated cell fraction. In these two cases, the contamination of the PBM cells with granulocytes might have been caused by the severity of the 5FU-associated toxicity or treatment with granulocyte...
colony-stimulating factor. In this respect, it is worthwhile to note that significant contamination of isolated mononuclear cells by granulocytes has been observed during storage of blood samples for 24 h (29, 30). Apparently, the integrity and density of the granulocytes are altered under these storage conditions because of the release of specific granule compartments (30, 31). In addition, poor recovery of mononuclear cells after 24 h of storage was observed because of platelet and leukocyte clumping (29, 32). Under such conditions the amount of isolated mononuclear cells might not be sufficient for proper analysis of DPD activity because a profound decrease in DPD activity was observed at low protein concentrations. Thus, the low DPD activity measured in PBM cells containing (immature) myeloid cells or that measured at a low protein concentration in the assay mixture is not indicative of heterozygosity for a mutant DPD allele.

Until now, fibroblasts often have been used for the diagnosis of patients with a complete DPD deficiency (26, 33). Furthermore, analysis of the DPD activity of family members of the index patients showed that obligate heterozygotes had lower DPD activity compared with those individuals possessing a wild-type genotype (26, 33). However, the overlapping range in DPD activities between controls and obligate heterozygotes precludes unambiguous detection of heterozygotes. Although it is now well established that the activity of DPD is generally lower in proliferating (malignant) cells than in resting (differentiated) cells (34), the DPD activity in fibroblasts was apparently not influenced by the degree of confluence of these fibroblasts.

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