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Research Article

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Familial Deficiency of Dihydropyrimidine Dehydrogenase

Biochemical Basis for Familial Pyrimidinemia and Severe 5-Fluorouracil-induced Toxicity

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Abstract

Severe neurotoxicity due to 5-fluorouracil (FUra) has previously been described in a patient with familial pyrimidinemia. We now report the biochemical basis for both the pyrimidinemia and neurotoxicity in a patient we have recently studied. After administration of a "test" dose of FUra (25 mg/m², 600 μ Ci [6-³H]FUra by intravenous bolus) to a patient who had previously developed neurotoxicity after FUra, a markedly prolonged elimination half-life (159 min) was observed with no evidence of FUra catabolites in plasma or cerebrospinal fluid and with 89.7% of the administered dose being excreted into the urine as unchanged FUra. Using a sensitive assay for dihydropyrimidine dehydrogenase in peripheral blood mononuclear cells, we demonstrated complete deficiency of enzyme activity in the patient and partial deficiency of enzyme activity in her father and children consistent with an autosomal recessive pattern of inheritance. Patients who are deficient in this enzyme are likely to develop severe toxicity after FUra administration.

Introduction

5-Fluorouracil (FUra)¹ is a pyrimidine antimetabolite that is widely used in the treatment of several common neoplasms including cancer of the gastrointestinal tract, ovary, and breast (1). The major toxic manifestations of this drug occur mainly in rapidly dividing tissues, such as bone marrow and the mucosal lining of the gastrointestinal tract. The pattern of toxicity is also dependent on the schedule of drug administration. Weekly administration by intravenous bolus typically causes depression of both white blood cell and platelet counts with stomatitis and diarrhea, while continuous intravenous administration more commonly produces less myelosuppression but more stomatitis, diarrhea, and skin changes (1, 2). Less frequent (~ 5%) toxicities include neurologic manifestations such as somnolence, upper motor neuron signs, cerebellar ataxia, and occasionally a cluster of symptoms and signs resembling "organic brain syndrome" (3, 4). The biochemical

basis for the neurologic toxicity secondary to FUra has not been elucidated. Previous reports have suggested that this toxicity was due to further metabolism of the FUra catabolite 2-fluoro- β -alanine to fluoroacetate and fluorocitrate, which are known neurotoxins (5, 6). However, actual formation of fluoroacetate and fluorocitrate from FUra has never been documented.

Recently, severe toxicity with cytopenias, stomatitis, diarrhea, and neurologic abnormalities was reported to develop in a patient being treated with FUra who also had familial pyrimidinemia and pyrimidinuria, suggesting that a genetic defect in pyrimidine catabolism may be the cause of the severe FUra toxicity (7). We now report a second patient with familial pyrimidinemia and pyrimidinuria who also developed severe toxicity after FUra administration. We have clarified the biochemical basis for the severe FUra toxicity observed, demonstrating altered FUra metabolism with a markedly prolonged plasma half-life and essentially complete absence of FUra catabolism. Using a sensitive assay for determining dihydropyrimidine dehydrogenase (DPD) activity in peripheral blood mononuclear cells, we observed that in contrast to cells from a control group who had detectable DPD activity there was complete deficiency of enzyme activity in the patient's cells and partial (< 50%) deficiency of enzyme activity in cells harvested from the patient's father and children, consistent with an autosomal recessive pattern of inheritance.

Methods

Case report

The patient is a 40-yr-old, white, married female who had been in excellent health throughout life until 3 mo before admission when she was admitted to the hospital for evaluation of a breast mass which on biopsy was shown to be infiltrating ductal carcinoma. She underwent a left-modified radical mastectomy (estrogen and progesterone receptors were negative) with axillary node dissection (9 of 16 axillary lymph nodes contained carcinoma). Initial laboratory studies including a chest x ray, chemistry profile, blood count, and urine analysis were within normal limits. Although there was no history of cancer or hereditary diseases, the family history was positive for consanguinity (marriage between either first or second cousins) in three preceding generations.

Adjuvant chemotherapy was begun 3 wk after surgery with cyclophosphamide (550 mg/m²), methotrexate (35 mg/m²), and FUra (550 mg/m²), to be administered intravenously every 21 d. The patient received cyclophosphamide (1,100 mg), methotrexate (70 mg), and FUra (1,100 mg) on day 1. 11 d later she was hospitalized with neutropenia (white blood cell count of 1,400 with 1% neutrophils and 1% bands) and fever. She defervesced on antibiotic therapy and was discharged home without complaints.

The second cycle of drugs was administered 26 d after day 1 of the initial cycle with reduced doses consisting of cyclophosphamide (600 mg), methotrexate (40 mg), and FUra (600 mg). 14 d later she returned to the clinic without complaints, but was observed to have a white blood cell count of 1,300.

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1. Abbreviations used in this paper: DPD, dihydropyrimidine dehydrogenase; FBAL, α -fluoro- β -alanine; FUH₂, dihydrofluorouracil; FUPA, α -fluoro-ureidopropionic acid; FUra, 5-fluorouracil.

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She received a third cycle of drugs on schedule but with a further dose reduction of cyclophosphamide (400 mg), methotrexate (30 mg), and FUra (400 mg). 14 d after beginning the third cycle the patient was rehospitalized with fever, neutropenia (white blood cell count of 1,500 with 1% neutrophils), and a 2-d history of "clumsy gait." She received antibiotics with resolution of the fever but her neurologic status continued to deteriorate: 5 d after admission she was unable to walk; on the 6th d she was unable to move her extremities; on the 7th d she was unable to speak. Studies obtained during this time included a negative toxic screen, a normal computed tomography (head) scan, and an electroencephalogram with generalized slowing compatible with an encephalopathy. Cerebrospinal fluid analysis demonstrated borderline elevated protein (46 mg/dl) with no evidence for infection as assessed by gram stain, india ink stain, or subsequent cultures. The patient was transferred to this institution where no infectious, toxic, or neoplastic cause for the neurologic deficit was found. A magnetic resonance imaging (head) scan was notable for evidence of diffuse periventricular, corpus callosum, and pontine hyperintensity consistent with demyelination. Of note were elevated pyrimidine levels in the patient's serum (demonstrated initially by HPLC and confirmed by GC-MS) with a uracil level of 1.510 $\mu\text{g/ml}$ (control range, 0.05 to 0.09 $\mu\text{g/ml}$) and a thymine level of 1.134 $\mu\text{g/ml}$ (control range, 0.08 to 0.44 $\mu\text{g/ml}$). Elevated uracil and thymine concentrations were also detected in urine and cerebrospinal fluid samples. 34 d after receiving the third cycle of drugs the patient was admitted to the Clinical Research Center where she underwent further evaluation to determine the basis for the pyrimidinemia and to determine if there was altered FUra metabolism that could explain the FUra-induced neurotoxicity.

The patient was subsequently treated with three additional cycles of chemotherapy with cyclophosphamide and methotrexate (without FUra), which she tolerated well. 2 mo after the onset of neurologic abnormalities, she regained the ability to speak. Over the succeeding 2 mo she slowly regained complete function of all four extremities. She has returned to her previous state of health with no neurologic deficits except for some memory loss for the time during which she was hospitalized. A follow-up magnetic resonance imaging scan 6 mo after the initial scan demonstrated evidence of remyelination in the previously affected areas. At the present time she remains in good health with no evidence of recurrent cancer.

Methods

Administration of [6-³H]FUra. The patient was studied with a protocol that had originally been designed to examine FUra pharmacokinetics and particularly the profile of FUra catabolites over time after administration of FUra by intravenous bolus (8). The protocol had previously been approved by the university's Institutional Review Board and Human Use Radiation Safety Committee. Informed consent was obtained before starting the study according to institutional guidelines.

[6-³H]FUra (26 Ci/mmol) was obtained from Moravsek Biochemicals (Brea, CA). Chemical and radiolabeled purity of [6-³H]FUra was > 99% as analyzed by HPLC. Radiolabeled FUra was initially passed through a 0.22- μm filter (Nalgene Industrial Dept., Nalge Co., Div. of Sybron Corp., Rochester, NY) and a 660- μCi aliquot was stored in a sterile vial at -20°C until use. Culture and pyrogen testing were performed by Laberco Testing Inc. (Roselle Park, NJ) to assure sterility before chemical use. Because of suspected toxicity to FUra, the patient received only a reduced "test" dose of FUra (25 mg/m^2) instead of the usual dose (550 mg/m^2). On the day of treatment 110% of the dose of "cold" FUra to be administered was mixed with an aliquot of the radiolabeled FUra using sterile technique. The calculated dose of FUra (patient's surface area 2.0 m^2 , total dose 50 mg) was administered by bolus injection over 1 min into the left arm with a free-flowing intravenous. The remaining 10% of the mixture was stored at -20°C and analyzed together with plasma, urine, and cerebrospinal fluid samples to determine any degree of degradation of drug in storage.

Collection of samples. A heparin lock was placed in the right arm of the patient (opposite from drug administration). 2-ml samples of blood were collected into the heparinized tubes at 0, 2, 5, 8, 12, 20, 30, 45, 60,

90, 120, 180, 240, and 360 min, and at 8 and 24 h postinjection. Collected blood samples were immediately placed on ice and centrifuged at 3,000 g for 10 min at 4°C . Plasma was separated and stored at -20°C until analysis.

Urine was collected in 2-h fractions for the initial 8 h and then 8-h fractions for the next 16 h. All urine samples were refrigerated during collection and 10-ml aliquots were frozen and stored at -20°C until analysis.

Cerebrospinal fluid samples (1.0 ml) were obtained by lumbar puncture at 1.5 and 6 h after drug administration. The samples were immediately stored at -20°C until analysis.

Analyses of the above samples were complete within 36 h after collection.

Analysis of FUra and metabolites. Unchanged FUra and its catabolites dihydrofluorouracil (FUH₂), α -fluoro-ureidopropionic acid (FUPA), and α -fluoro- β -alanine (FBAL) were quantitated as described previously (9). Authentic standards of FUra, FUH₂, FUPA, and FBAL had been previously obtained from Hoffmann-La Roche, Inc. (Nutley, NJ). Initially, samples were passed through a 0.22- μm filter (Arco, Gelman Sciences, Ann Harbor, MI) and then aliquots of plasma (100 μl), urine (10–200 μl), and cerebrospinal fluid (100 μl) were analyzed with a high performance liquid chromatograph (model 1084B; Hewlett-Packard Co., Avondale Div., Avondale, PA) equipped with an automatic injector and a variable wavelength ultraviolet detector. Analysis was accomplished with two (25 \times 0.4 cm) 5- μm columns (RP-18; IBM Instruments, Inc., Poughkeepsie, NY) in tandem. Elution was carried out isocratically at 1.0 ml/min with a mobile phase consisting of 5 mM tetrabutylammonium hydrogen sulfate and 1.5 mM potassium phosphate buffer (pH 8). Timed fractions (0.5 or 1.0 min) were collected into 7-ml plastic scintillation vials using a fraction collector (Redirac 2122; LKB Instruments, Rockville, MD) that was automated for timed fraction collection as had earlier been described (9). The radioactivity in each fraction was quantitated with a liquid scintillation counter (LS-5801; Beckman Instruments, Inc., Irvine, CA).

Pharmacokinetic analysis. Pharmacokinetic parameters were calculated using noncompartmental analysis based on statistical moment theory (10). Elimination half-life of unchanged drug was obtained by linear regression analysis of the kinetics terminal points (least-squares method). The area under the curve was calculated from time zero to the last point using the trapezoidal rule with extrapolation to infinity. The total plasma clearance was calculated by dividing the dose by the area under the curve.

Assay of DPD activity. Blood (25 ml) was removed from a peripheral vein into a heparinized syringe, and then centrifuged through a Ficoll-Hypaque discontinuous gradient to separate mature erythroid blood cells from peripheral blood mononuclear cells. The peripheral blood mononuclear cell fraction was removed and mixed with 1 ml of 5 mM sodium phosphate buffer (pH 7.5). The cells were then lysed by freeze thawing and sonicating five times for periods of 10 s while in an ice bath. After removal of debris by centrifugation (20,000 g for 30 min) the supernatant was assayed for DPD using a modification of a method described by Marsh and Perry (11) and assayed for protein concentration by the method of Lowry et al. (12). Aliquots (730 μl) of the supernatant were incubated at 37°C for varying intervals over 180 min in the presence of 250 μM NADPH and 20 μM [6-³H]FUra (3.5 $\mu\text{Ci}/\mu\text{mol}$). At the end of the incubation time the sample was immediately frozen in dry ice/acetone and then (within 30 min) analyzed for the presence of FUra and FUra catabolites by HPLC with collection of fractions and quantitation of radioactivity as described above. The enzyme activity was expressed as nanomoles of total catabolites formed (FUH₂, FUPA, and FBAL) per hour per milligram of protein.

Results

Kinetics of FUra and metabolites in plasma. After administration of radiolabeled FUra by intravenous bolus there was no

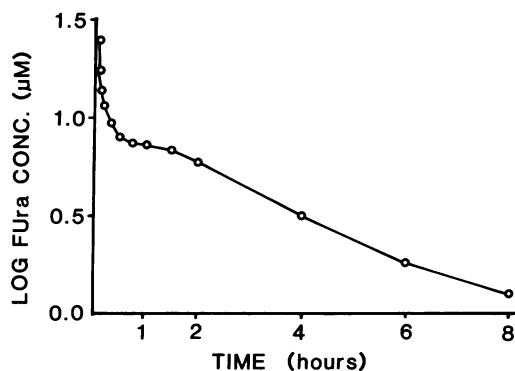


Figure 1. Plasma concentration of Fura (○) in proband over 24 h after intravenous bolus of 25 mg/m² Fura. (Note that there was no detectable evidence of Fura catabolite at any of the time points examined.)

evidence of Fura catabolites (or other metabolites) in plasma at any of the 15 time points examined over 24 h. Note that the limit of sensitivity for FUH₂, FUPA, and FBAL was ~ 0.2 μM. Fig. 1 demonstrates that Fura was detectable at each of the time points with a β -phase elimination half-life for Fura of ~ 159 min. The total plasma clearance of Fura was 70 ml/min per m².

HPLC analysis of urine for Fura and metabolites. Because of the possibility that Fura catabolites may have been formed and rapidly cleared into the urine, we examined the urine for the presence of Fura and metabolites. Fig. 2 depicts the urinary profile observed during the 24 h after the intravenous bolus of radiolabeled Fura. Note that within each of the six collection intervals, Fura accounted for most of the excreted radioactivity, with FBAL and the FUH₂-FUPA fraction accounting for only a small proportion of the excreted radioactivity. Cumulatively over the 24-h period, 97.4% of the 50 mg of radiolabeled Fura administered by intravenous bolus was recovered in the urine with 44.85 mg (89.7%) as Fura, 3.02 mg (6.03%) as FBAL, and 0.81 mg (1.62%) as FUH₂ and FUPA. No other metabolites were detected in the urine. Essentially all of the radioactivity injected on the HPLC column was recovered (> 99%).

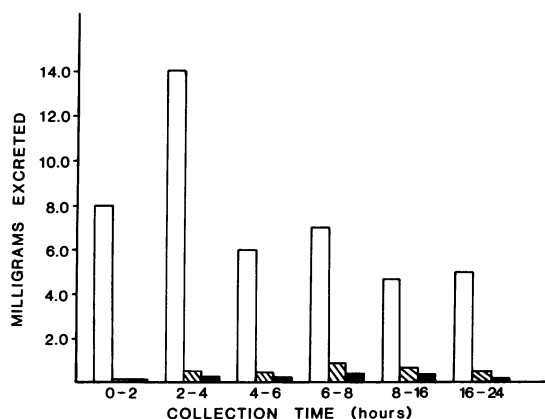


Figure 2. Profile of unchanged drug (□), FBAL (■), and FUH₂ + FUPA (▨) excreted during each of the collection intervals over the 24 h after drug (Fura) administration.

Table I. DPD Activity in Peripheral Blood Mononuclear Cells from Controls, Patient, and Family Members

| Subject | DPD activity nmol/h/mg protein* | Percent of normal |
|---------------------------|------------------------------------|-------------------|
| Control (n = 5; \pm SD) | 279 \pm 21 | 100 |
| I-1 Father† | 75 | 26.9 |
| II-4 Brother | 275 | 98.7 |
| II-5 Proband‡ | ND | 0 |
| III-1 Niece | 308 | 110.6 |
| III-2 Nephew | 126 | 45.2 |
| III-5 Daughter | 66 | 23.7 |
| III-6 Son | 88 | 31.6 |

* DPD activity was quantitated as described in Methods and represent nanomoles of Fura catabolites formed per hour per milligram protein.

† The roman numeral refers to the generation, and the arabic number to the specific member of that generation as shown in Fig. 3.

‡ Examined two separate times.

^{||} ND, Not detectable.

HPLC analysis of cerebrospinal fluid for Fura and metabolites. Unchanged Fura was detected in cerebrospinal fluid at 1.5 h (0.29 μM) and at 6 h (0.87 μM) after Fura administration. There was no evidence for any other Fura metabolites in the cerebrospinal fluid and specifically no radioactivity was detected co-eluting with authentic standards of fluorocitrate and fluoroacetate. Recovery of radioactivity was > 99% for both samples with essentially all of the radioactivity injected onto the HPLC column being recovered as the parent drug.

Presence of DPD activity in peripheral blood mononuclear cells from patient and family members. Table I lists the DPD activity detected in peripheral blood mononuclear cells isolated from the proband and her family members. The relationship of the various family members is further illustrated in the pedigree in Fig. 3, which also shows which of the family members were deceased, which of the family members who were alive were tested, and the relative presence of DPD activity. The proband had no evidence of enzyme activity in her peripheral blood mononuclear cells on either occasion that she was examined. The proband's son and daughter had DPD

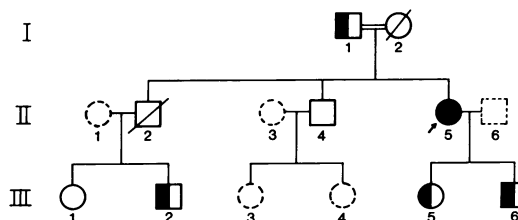


Figure 3. Pedigree of a family showing inheritance of a defect in DPD activity. The proband is indicated by an arrow, deceased family members by a diagonal line thru the symbol, and subjects who were alive but not examined by a dashed symbol. Consanguineous marriage is shown by a double line connecting male and female symbols. Half-shaded symbols indicate partial deficiency of DPD activity (< 50% control). Nonshaded symbols indicate control levels of DPD activity. The fully shaded symbol represents complete deficiency of DPD activity.

activities of 31.6 and 23.7% of control values, respectively. Note that there was no difference between teenage controls (corresponding to proband's children) and adult controls (data not shown). The proband's father also had a partial deficiency of DPD activity of 26.9% of control values. In contrast, the proband's only living sib, a 45-yr-old brother, had no evidence for deficiency of DPD activity, with activity in the same range as that of controls. Two children of her late brother were also examined, with the nephew having a DPD activity equal to 45.2% of control and the niece having no evidence of deficiency of DPD activity.

Discussion

This study provides further insight into a recently described syndrome in which severe FUra-induced toxicity with neurologic dysfunction was shown to be associated with familial pyrimidinemia and pyrimidinuria. The present study describes a patient who had an almost identical presentation: a previously unremarkable medical history, subsequent diagnosis of breast cancer with mastectomy, and adjuvant chemotherapy with cytoxan, methotrexate, and FUra, who then develops unexpected toxicity including severe neurologic toxicity. Both the patient we have described and the one described previously (7) are different from those with previously described syndromes of familial pyrimidinemia and pyrimidinuria described in the pediatric literature who characteristically have baseline neurologic dysfunction with mental retardation, developmental abnormalities, and various other neurologic maladies (13–15).

We have demonstrated that there is a deficiency in the enzyme that converts (chemically reduces) the pyrimidine base (e.g., uracil or thymine) to a dihydropyrimidine. Using a specific and sensitive assay for this enzymatic step, we have demonstrated (see Table I and Fig. 3) complete deficiency in the affected patient (proband) with evidence of partial deficiency in the patient's children, her nephew, and her father. This coupled with the notable history of consanguinity in this family suggests an autosomal recessive pattern of inheritance.

In addition to elucidating this novel inborn error of metabolism we have also investigated the effect of this enzymatic deficit on fluoropyrimidine metabolism in particular. Using techniques we have recently described for studying fluoropyrimidine catabolism in cellular (9) and clinical studies (8), we have demonstrated a marked alteration in FUra pharmacokinetics after administration of a "test dose" of FUra. Note that this dose is approximately one-twentieth of the FUra dose typically used (500–600 mg/m²) in most regimens (1). We have recently reported (8) in detailed studies of fluoropyrimidine catabolism in man that after administration of [6-³H]-FUra (600 μ Ci, 550 mg/m²), unchanged FUra was rapidly cleared from plasma (594 \pm 198 ml/min per m²) with an elimination half-life of 13 \pm 7 min. Even at the earliest time point (2.0 min) there was evidence for pyrimidine catabolite formation (with FUH₂ and FUPA being the major metabolites detected at the early times and FBAL being the major metabolite present after 2 h). In contrast, in the patient we have described in the present study there was minimal evidence of FUra catabolism. Indeed one would have expected the relatively small amount of administered FUra to have been rapidly metabolized and cleared from the plasma. Instead, as shown in Fig. 1,

FUra was detectable in plasma at all time points examined with a markedly prolonged apparent elimination half-life of 159 min. FUra catabolites were not detected in the plasma of this patient. Although there was evidence of fluoropyrimidine catabolites in the urine, this was minimal (see Fig. 2), and in contrast to the earlier study (8), where FUra was a minor (9.8 \pm 1.6%) component of the excreted radioactivity in the urine over 24 h, FUra was found to be the major component in the urine in the present study with 89.7% of the administered FUra being recovered unchanged in the urine. Table II summarizes the difference in FUra pharmacokinetics between the patient with a DPD deficiency and a patient control group we previously studied (8) with no evidence of DPD deficiency.

Of particular importance to understanding FUra-induced neurotoxicity was the absence of fluoropyrimidine catabolites in the cerebrospinal fluid with specifically no evidence of radioactivity co-eluting with fluorocitrate or fluoroacetate. This coupled together with the absence of FBAL, which would be expected before further metabolism to fluorocitrate and fluoroacetate (5, 6), suggest that accumulation of these FUra catabolites is an unlikely mechanism by which FUra-induced neurotoxicity is produced. The possibility exists that prolonged exposure to relatively high concentrations of FUra may lead to metabolic abnormalities in the neural tissue. Since DNA would not be expected to replicate in nervous tissue, it is unlikely that anabolism to 5-fluoro-2'-deoxyuridine 5'-monophosphate, a known inhibitor of thymidylate synthase, or incorporation of 5-fluoro-2'-deoxyuridine 5'-triphosphate into DNA, would have any substantial effect. Conversely it is possible that increased incorporation of FUra into cellular RNA might result in a metabolic abnormality, perhaps leading to defective myelin production and possibly other neuropathologic consequences.

In summary, the present study has demonstrated that deficiency of DPD activity is the probable cause of the elevated pyrimidine levels observed in a recently described (7) syndrome of familial pyrimidinemia and pyrimidinuria. Our

Table II. Comparison of FUra Pharmacokinetics in Presence and Absence of DPD Deficiency

| | $t_{1/2}$ * | Cl | Unchanged FUra recovered in urine in 24 h (% of dose) |
|-------------------------------|-------------|-----------------------|---|
| | min | ml/min/m ² | |
| Proband* | 159 | 70 | 89.7 |
| Patient [§] controls | 13 \pm 7 | 594 \pm 198 | 9.8 \pm 1.6 |

* $t_{1/2}$, Apparent elimination half-life; Cl, total plasma clearance calculated by dividing the dose by the area under the curve.

[†] The proband had previously experienced severe toxicity after a typical clinical dose of FUra. The pharmacokinetic data presented here is after an intravenous bolus "test dose" of 25 mg/m² FUra.

[§] These controls consisted of 10 cancer patients who were previously studied with an identical protocol (see reference 8) except that the pharmacokinetic data is after a "typical" intravenous bolus dose of 500 mg/m² FUra. Values are expressed as mean \pm SD.

^{||} Approximately 80% of the administered drug was detected in the urine in 24 h as FUra catabolites with the major fraction consisting of FBAL, and minor fraction consisting of FUH₂ and FUPA (see reference 8).

study suggests that this syndrome is inherited as an autosomal recessive trait. When an individual such as our patient who is completely deficient in DPD activity (a probable homozygote for a gene resulting in defective DPD activity compared with the "wild type") is given FUra, an altered pattern of FUra metabolism occurs, resulting in a markedly prolonged exposure to FUra, and in turn increased toxicity. Further, the occurrence of FUra-induced neurologic toxicity appears not to result from formation of fluoropyrimidine catabolites (fluorocitrate, fluoroacetate) as has previously been suggested (5, 6), but instead may be due to prolonged exposure to relatively high concentrations of FUra in the central nervous system, possibly resulting in increased incorporation of FUra into RNA. Lastly, the presence of a partial deficiency of DPD in the father, children, and nephew of the patient (probable heterozygotes) raises the possibility that such individuals may also have altered FUra metabolism if given FUra. Individuals who are heterozygotes for this gene may be at increased risk to develop FUra toxicity compared with individuals not carrying this gene (although with less toxicity than those individuals who are homozygotes for this gene). Future studies should address this possibility.

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