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# IMPACT OF DIETARY FOLIC ACID ON REDUCED FOLATES IN MOUSE PLASMA AND TISSUES

## RELATIONSHIP TO DIDEAZATETRAHYDROFOLATE SENSITIVITY

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**Abstract**—To investigate the role of dietary folic acid in dideazatetrahydrofolate (DDATHF) sensitivity, reduced folates were estimated in plasma and tissue of mice following dietary depletion and repletion. Previous studies showed that DDATHF, a new folate antagonist targeted against glycinamide ribonucleotide transformylase, produced unexpectedly severe toxicity in humans compared with mice. However, toxicity in the animal model also became pronounced upon the removal of folic acid from the diet. Further, modest dietary restoration of folic acid in the drinking water showed that toxicity could be alleviated while antitumor activity was maintained. To investigate the role of dietary folic acid levels on tissue folates in this system, all the natural reduced folates were evaluated by a ternary complex based assay in mice placed on folic acid deplete and replete diets. After 2 weeks on a folic acid deplete diet, total plasma folate had decreased by 85%, whereas red blood cell, liver, and intestinal folate fell by only 50%. Repletion of folic acid in the drinking water at a low level (0.0003%) caused partial restoration of reduced folates, while a higher repletion level (0.003%) resulted in restoration to control levels or above. Administration of folic acid and leucovorin by oral gavage to DDATHF-treated mice resulted in elevation of tissue folates in mice maintained on folic acid deplete and replete diets. Relatively high levels of folic acid were present in plasma following oral gavage of folic acid, while essentially no [*S*]5-formyltetrahydrofolate was observed after leucovorin. Reduced folate pools in a subcutaneously implanted mouse mammary adenocarcinoma responded more extensively to dietary folic acid depletion than folate pools in liver. Likewise, these pools were more sensitive to restoration by folic acid or leucovorin. This greater reduced folate response of tumor versus normal tissue, if confirmed in other systems, suggests a possible basis for selective antitumor activity.

*Key words:* folic acid; folate deficiency; dideazatetrahydrofolate; leucovorin; mouse tissue folate

The folate antagonist MTX§ has made a long-standing contribution to cancer chemotherapy [1]. However, drug resistance, as well as lack of efficacy in some types of cancer, has led to consideration of other folate antagonists, particularly those that target enzymes other than dihydrofolate reductase. The glycinamide ribonucleotide formyltransferase inhibitor DDATHF [2, 3] is one such drug that has shown strong antitumor activity in animal studies [4]. However, when clinical trials in humans were conducted, unexpectedly severe thrombocytopenia and anemia prevented drug escalation to levels achievable in mice [5, 6]. The basis for the lesser toxicity in mice was shown to be related to dietary FA, because when mice were placed on a folate deplete diet they became much more like humans with regard to the toxic effects of DDATHF [7]. Further, FA repletion in drinking water resulted in diminished toxicity and, by adjustment to the proper level, this could be accomplished without extensive loss of antitumor activity. To examine the extent to which these changes in dietary FA can alter plasma and tissue reduced folates and, hence, potentially play a role in DDATHF therapeutics, reduced folate pools were evaluated in mice placed on FA deplete and replete diets.

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§ Abbreviations: MTX, methotrexate; DDATHF, dideazatetrahydrofolate or lometrexol; FA, folic acid; 5-$CHOFH_4$, 5-formyltetrahydrofolate or leucovorin; FdUMP, fluorodeoxyuridine monophosphate; RBC, red blood cell; $CH_2FH_4$, 5,10-methylenetetrahydrofolate; $FH_4$, tetrahydrofolate; $FH_2$, dihydrofolate; 5-$CH_3FH_4$, 5-methyltetrahydrofolate; 10-$CHOFH_4$, 10-formyltetrahydrofolate; GAR, glycinamide ribonucleotide; and FPGS, foly-polyglutamate synthetase.

### MATERIALS AND METHODS

*Materials.* DDATHF for these studies was synthesized in the laboratory of C. Shih (Lilly Research Laboratories, Indianapolis, IN). [$^3$H]-FdUMP was purchased from Moravek Biochemicals (Brea, CA). FA, [*R,S*]5-$CHOFH_4$, NADPH and other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO). Thymidylate synthase (3.7 U/mg protein) was purified from an *Escherichia*

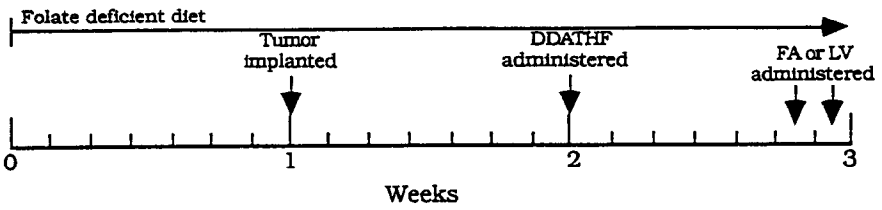


Fig. 1.

*coli* strain that overproduces *Lactobacillus casei* thymidylate synthase [8]. The *Escherichia coli* strain was a gift from D. Santi (University of California, San Francisco). 5,10-Methylenetetrahydrofolate reductase (0.62 U/mg protein), 10-formyltetrahydrofolate deacylase (1.1 mU/mg protein), and dihydrofolate reductase (1.25 U/mg protein) were purified from pig liver [9], beef liver [10], and methotrexate-resistant *L. casei* [11], respectively, as described previously.

*Animal treatment.* C3H mice were maintained on standard or FA deplete chow for 2 weeks. The FA deplete mice were then supplemented with 0.0003% or 0.003% FA in their drinking water for the next 5 days. All groups drank an average of 4 mL/mouse/day. Six mice from each group were killed to obtain blood and tissues. In a separate experiment (Fig. 1), mice maintained on FA deplete and replete chow for 3 weeks were implanted with C3H mammary adenocarcinoma at the beginning of week 2. At the end of week 2, mice were injected intravenously with DDATHF (30 mg/kg). On days 6 and 7 following this injection, either leucovorin (30 mg/kg) or FA (30 and 100 mg/kg) was administered by oral gavage. Four hours after the last oral folate dose, three mice from each group were killed to obtain plasma, liver, and tumor.

*Tissue preparation.* Whole blood was collected in EDTA-containing tubes and centrifuged immediately at 400 *g* for 5 min at 4°. The plasma obtained was diluted with an equal volume of a cold 50 mM Tris–HCl buffer (pH 7.4) containing 100 mM sodium ascorbate and 1 mM EDTA and stored at −70°. Pelleted RBCs were washed with cold PBS (pH 7.4) containing 0.02% glucose, recentrifuged, and stored at −70°. Liver, tumor, and intestine were removed from mice and washed with cold PBS before storage at −70°. For routine analysis of folates, plasma and RBCs were diluted into a 50 mM Tris–HCl buffer containing 50 mM sodium ascorbate and 1 mM EDTA, placed in a boiling water bath for 5 min, and centrifuged to remove precipitated protein. Liver, tumor, and scraped mucosa from intestine were each homogenized in cold 50 mM Tris–HCl buffer containing 50 mM sodium ascorbate and 1 mM EDTA and centrifuged at 4° to remove cell debris. An aliquot of the supernatant was used for soluble protein determination by the method of Bradford [12]. The remainder of the supernatant was immediately placed in a boiling water bath for 5 min and centrifuged to remove precipitated protein. The resultant supernatants were used for folate estimation. Reference folates were stable under these conditions with routine recovery in the range of 70–95%. Because of concern regarding interconversion in boiled tissue extracts. $CH_2FH_4$ and $FH_4$ are reported as a combined pool. Likewise, analysis solutions for FA contained sufficient dihydrofolate reductase to reduce $FH_2$ as well, so these two folates are also reported as the sum even though it is unlikely that significant $FH_2$ is present under the conditions used.

*Estimation of folates.* The ternary complex assay is based upon enzymatic cycling of reduced folates to $CH_2FH_4$ followed by entrapment into a stable ternary complex with excess thymidylate synthase and [$^3$H]FdUMP. Methods have been described previously for the estimation of $CH_2FH_4$, $FH_4$, 5-$CH_3FH_4$, 10-$CHOFH_4$, [*S*]5-$CHOFH_4$, $FH_2$, and FA using this approach [13]. Typically, reaction mixtures contained 20 mU thymidylate synthase and 125 nM [$^3$H]FdUMP (20 Ci/mmol) in 200 μL of a buffer prepared from 50 mM Tris–HCl (pH 7.4), 50 mM sodium ascorbate, and 1 mM EDTA. Additional cofactors and enzymes were added as necessary for cycling each reduced folate to $CH_2FH_4$. Complex formation was allowed to proceed at 25° for 30 min. Addition of 1% SDS and boiling for 10 min were used to stop reactions. Aliquots (25 μL) were eluted from 400-μL minicolumns of Sephadex G-25 by centrifugation to separate tritiated complexes from free [$^3$H]FdUMP. Bound radioactivity was determined by scintillation counting. The practical limit of detection for $CH_2FH_4$ and other folates under these conditions was 7 fmol [13].

## RESULTS

To determine the impact of dietary FA depletion, reduced folate pools were estimated in plasma, RBCs, liver and intestine of mice placed on an FA deplete diet for 2 weeks. It can be seen in Table 1 that total folate, based on the sum of all natural folates, was diminished by more than 6-fold in plasma. While 5-$CH_3FH_4$, the predominate plasma folate, was depleted most extensively, the $CH_2FH_4$ + $FH_4$ pool, which was present at significant levels even in control animals, also responded to dietary FA depletion.

As expected, RBC folates were predominately 5-$CH_3FH_4$. However, significant amounts of $CH_2FH_4$ + $FH_4$ and 10-$CHOFH_4$ could also be detected. Total folate levels in RBCs did not respond as dramatically as plasma to dietary FA depletion.

Table 1. Effects of dietary folic acid on folates in mouse plasma and tissues

| Tissue | Diet | Tissue folates | | | | |
|---|---|---|---|---|---|---|
| | | 5-$CH_3FH_4$ | $CH_2FH_4 + FH_4$ | $FA + FH_2$ | 10-$CHOFH_4$ | Total |
| Plasma (pmol/mL) | Control | 74 ± 13 | 12 ± 5 | 2 ± 1 | <1 | 88 ± 18 |
| | Folate deplete* | 8 ± 2 | 3 ± 2 | <1 | <1 | 13 ± 3 |
| | 0.0003% added† | 35 ± 10 | 5 ± 3 | <1 | 2 ± 1 | 43 ± 15 |
| | 0.003% added† | 91 ± 14 | 24 ± 9 | 16 ± 5 | 11 ± 5 | 142 ± 32 |
| RBCs (pmol/mL) | Control | 1215 ± 43 | 21 ± 5 | 7 ± 3 | 75 ± 15 | 1317 ± 45 |
| | Folate deplete | 665 ± 39 | 9 ± 4 | 7 ± 3 | 44 ± 9 | 725 ± 35 |
| | 0.0003% added | 797 ± 59 | 8 ± 1 | 7 ± 2 | 71 ± 8 | 883 ± 68 |
| | 0.003% added | 1108 ± 42 | 24 ± 5 | 7 ± 2 | 96 ± 7 | 1235 ± 46 |
| Liver (pmol/mg protein) | Control | 125 ± 16 | 293 ± 27 | 6 ± 3 | 49 ± 13 | 478 ± 23 |
| | Folate deplete | 87 ± 9 | 141 ± 19 | 5 ± 2 | 7 ± 2 | 239 ± 17 |
| | 0.0003% added | 123 ± 12 | 278 ± 45 | 31 ± 14 | 32 ± 11 | 463 ± 53 |
| | 0.003% added | 142 ± 18 | 560 ± 180 | 21 ± 11 | 63 ± 13 | 619 ± 116 |
| Intestine (pmol/mg protein) | Control | 12 ± 5 | 21 ± 2 | 7 ± 4 | 45 ± 12 | 96 ± 12 |
| | Folate deplete | 4 ± 2 | 18 ± 5 | 3 ± 1 | 12 ± 5 | 41 ± 6 |
| | 0.0003% added | 10 ± 3 | 29 ± 2 | 3 ± 1 | 14 ± 5 | 73 ± 5 |
| | 0.003% added | 19 ± 4 | 59 ± 3 | 50 ± 27 | 20 ± 6 | 154 ± 28 |

Folate determinations represent the means ± SEM from duplicate assays of six tissues samples (total of 12 estimations).
* Mice were given a folate deplete diet for 2 weeks.
† Folic acid (0.0003% or 0.003%) was added to the drinking water of the folate deplete mice for 5 days.

Table 2. Effects of leucovorin and folic acid rescue on folate pools in plasma of mice treated with DDATHF

| Diet | Treatment | Plasma folate concentration (pmol/mL) | | | | |
|---|---|---|---|---|---|---|
| | | 5-$CH_3FH_4$ | $CH_2FH_4$ + $FH_4$ | FA + $FH_2$ | 10-$CHOFH_4$ | Total |
| Folate replete | Control | 76 ± 2 | 14 ± 1 | <1 | <1 | 92 ± 3 |
| | DDATHF* | 76 ± 2 | 11 ± 1 | 2 ± 1 | <1 | 90 ± 2 |
| | DDATHF + LV† | 131 ± 13 | 82 ± 9 | 10 ± 3 | 3 ± 2 | 227 ± 23 |
| | DDATHF + FA† | 168 ± 16 | 144 ± 3 | 111 ± 10 | 10 ± 5 | 433 ± 21 |
| | DDATHF + FA‡ | 167 ± 15 | 156 ± 13 | 412 ± 65 | 23 ± 6 | 758 ± 85 |
| Folate deplete | Control | 6 ± 2 | 2 ± 2 | <1 | <1 | 7 ± 3 |
| | DDATHF* | 12 ± 2 | <1 | <1 | <1 | 12 ± 2 |
| | DDATHF + LV† | 100 ± 11 | 50 ± 1 | 8 ± 3 | 4 ± 2 | 160 ± 16 |
| | DDATHF + FA† | 83 ± 28 | 48 ± 22 | 255 ± 78 | <1 | 387 ± 65 |
| | DDATHF + FA‡ | 86 ± 4 | 68 ± 7 | 528 ± 73 | 9 ± 3 | 691 ± 73 |

Folate estimates represent the means ± SEM from duplicate assays of three plasma samples (total of 6 estimations).
* For all treatments, DDATHF was administered i.v. at a dose of 30 mg/kg.
† Leucovorin and folic acid were administered by oral gavage at a dose of 30 mg/kg on days 6 and 7 after DDATHF.
‡ Folic acid was administered by oral gavage at a dose of 100 mg/kg on days 6 and 7 after DDATHF.

They were diminished only by approximately one-half. Total folate in liver and intestinal tissue responded to the FA deplete diet in a manner similar to RBCs in that they were also diminished by approximately one-half. Comparison between tissues shows that whether on the control or FA deplete diet total liver folate was five to six times higher than intestinal folate. When individual folates were compared between these two tissues, the liver was generally richer in the more reduced folate forms, 5-$CH_3FH_4$ and $CH_2FH_4$ + $FH_4$.

Dietary FA was repleted at two levels by adding it to the drinking water for a period of 5 days at the end of the 2-week depletion period. The higher level, 0.003%, was the level used previously to overcome DDATHF toxicity while maintaining antitumor activity [7]. A lower level, 0.0003%, was used to test the sensitivity of folates to more modest dietary FA changes. Table 1 shows that plasma folates responded to drinking water FA repletion in a dose-dependent manner. At the higher level, 10-$CHOFH_4$ and FA + $FH_2$ were readily detectable even though they were negligible in plasma of control animals. RBCs, liver, and intestinal folates also responded in a dose-dependent manner to FA repletion in the drinking water, and generally, the distribution of individual pools was about the same as in control animals.

To examine the impact of DDATHF on reduced folate pools and their response to rescue by FA and 5-$CHOFH_4$, mice maintained on FA replete and deplete diets were injected with a relatively high dose of DDATHF (30 mg/kg) 5 days before receiving FA at 30 or 100 mg/kg doses daily for 2 days by oral gavage. A 30 mg/kg dose of [*R*,*S*]5-$CHOFH_4$ was also administered by the same route for comparison. It can be seen in Table 2 that plasma folates of mice receiving FA deplete or replete diets were relatively unaffected by DDATHF. 5-$CHOFH_4$ administration to drug-treated animals caused elevation of total plasma folate but only about to one-half the extent of FA at the same total dose. This may be attributed to the fact that only [*S*]5-$CHOFH_4$ is active, and the [*R*,*S*] mixture was administered. Thus, total folate response was approximately proportional to active folate administered in both cases. The total amount by which folates were elevated by 5-$CHOFH_4$ or FA did not depend upon folate status prior to administration. Folate deplete mice gained about the same amount of total folate as did replete animals. The FA response was dose dependent, but the 3.3-fold increase in dose resulted in only approximately a 2-fold elevation in total folate. Further, most of the increase could be accounted for by FA + $FH_2$, which is likely primarily FA, with relatively little change in fully reduced folate metabolites. On the other hand, no parent compound, [*S*]5-$CHOFH_4$, was detectable in plasma after [*R*,*S*]5-$CHOFH_4$ administration.

It can be seen in Table 3 that total reduced folate response to dietary FA depletion in liver was also generally unaffected by DDATHF. Further, administration of [*R*,*S*]5-$CHOFH_4$ and both doses of FA raised the level of folates in the liver by about the same amount in folate replete animals as in animals on the folate deplete diet. The 5-$CH_3FH_4$ and $CH_2FH_4$ + $FH_4$ pools that already accounted for most of the liver folate became most extensively elevated in response to both 5-$CHOFH_4$ and FA.

To evaluate the response of tumor tissue folates, a mouse mammary adenocarcinoma was implanted subcutaneously in animals 1 week prior to DDATHF administration. It can be seen in Table 4 that the impact of an FA deplete diet on tumor reduced folates was much greater than the effect on liver reduced folates (see Table 3). In tumor tissue, dietary FA depletion resulted in a 5-fold depletion of total folate, whereas in liver depletion was less than 2-fold. Likewise, total folates were elevated 4- to 5-fold following administration of either folate source in tumors of animals on the FA deplete diet, but only about 1.2-fold in replete animals. While this same trend could be seen in liver (Table 3), the magnitude was much less (1.7-fold vs 1.5-fold). Like liver, the same individual folates as already present in tumor tissue generally responded most to 5-

Table 3. Effects of leucovorin and folic acid rescue on folate pools in liver of mice treated with DDATHF

| Diet | Treatment | Liver folate concentration (pmol/mg protein) | | | | |
|---|---|---|---|---|---|---|
| | | 5-$CH_3FH_4$ | $CH_2FH_4$ + $FH_4$ | FA + $FH_2$ | 10-$CHOFH_4$ | Total |
| Folate replete | Control | 161 ± 21 | 352 ± 37 | <1 | 9 ± 9 | 522 ± 51 |
| | DDATHF* | 212 ± 51 | 438 ± 76 | <1 | 3 ± 3 | 652 ± 122 |
| | DDATHF + LV† | 248 ± 56 | 473 ± 22 | 6 ± 6 | <1 | 727 ± 70 |
| | DDATHF + FA† | 196 ± 29 | 590 ± 36 | <1 | <1 | 785 ± 18 |
| | DDATHF + FA‡ | 158 ± 16 | 615 ± 25 | 9 ± 9 | 36 ± 18 | 818 ± 35 |
| Folate deplete | Control | 101 ± 39 | 167 ± 44 | <1 | <1 | 268 ± 78 |
| | DDATHF* | 129 ± 33 | 115 ± 30 | <1 | <1 | 244 ± 61 |
| | DDATHF + LV† | 216 ± 58 | 196 ± 59 | <1 | <1 | 412 ± 117 |
| | DDATHF + FA† | 201 ± 45 | 294 ± 111 | 8 ± 8 | <1 | 504 ± 147 |
| | DDATHF + FA‡ | 222 ± 55 | 235 ± 27 | 5 ± 5 | 10 ± 10 | 472 ± 30 |

Folate estimates represent the means ± SEM from duplicate assays of three liver samples (total of 6 estimations).
* For all treatments, DDATHF was administered i.v. at a dose of 30 mg/kg.
† Leucovorin and folic acid were administered by oral gavage at a dose of 30 mg/kg on days 6 and 7 after DDATHF.
‡ Folic acid was administered by oral gavage at a dose of 100 mg/kg on days 6 and 7 after DDATHF.

Table 4. Effects of leucovorin and folic acid rescue on folate pools in tumors of mice treated with DDATHF

| Diet | Treatment | Tumor folate concentration (pmol/mg protein) | | | | |
|---|---|---|---|---|---|---|
| | | 5-$CH_3FH_4$ | $CH_2FH_4$ + $FH_4$ | FA + $FH_2$ | 10-$CHOFH_4$ | Total |
| Folate replete | Control | 44 ± 12 | 115 ± 24 | 16 ± 9 | <1 | 175 ± 28 |
| | DDATHF* | 44 ± 14 | 124 ± 24 | 12 ± 7 | <1 | 182 ± 31 |
| | DDATHF + LV† | 102 ± 20 | 75 ± 9 | 5 ± 3 | 37 ± 4 | 218 ± 26 |
| | DDATHF + FA† | 88 ± 5 | 96 ± 10 | 14 ± 8 | 19 ± 1 | 216 ± 16 |
| | DDATHF + FA‡ | 87 ± 8 | 65 ± 1 | 19 ± 10 | 48 ± 8 | 218 ± 19 |
| Folate deplete | Control | 2 ± 2 | 23 ± 3 | 10 ± 5 | <1 | 34 ± 2 |
| | DDATHF* | 9 ± 4 | 27 ± 2 | 7 ± 7 | 2 ± 2 | 45 ± 3 |
| | DDATHF + LV† | 73 ± 13 | 55 ± 11 | 15 ± 10 | 14 ± 2 | 156 ± 10 |
| | DDATHF + FA† | 78 ± 11 | 86 ± 26 | 10 ± 6 | 13 ± 6 | 186 ± 27 |
| | DDATHF + FA‡ | 101 ± 5 | 72 ± 10 | 14 ± 4 | 9 ± 5 | 196 ± 24 |

Folate estimates represent the means ± SEM from duplicate assays of three tumor samples (total of 6 estimations).
* For all treatments, DDATHF was administered i.v. at a dose of 30 mg/kg.
† Leucovorin and folic acid were administered by oral gavage at a dose of 30 mg/kg on days 6 and 7 after DDATHF.
‡ Folic acid was administered by oral gavage at a dose of 100 mg/kg on days 6 and 7 after DDATHF.

$CHOFH_4$ and FA. However, the 10-$CHOFH_4$ pool, which was very low in tumor of both folate deplete and replete animals before folate restoration, responded more extensively than other folates to both 5-$CHOFH_4$ and FA.

## DISCUSSION

The level and distribution of individual reduced folates would be expected to be important factors in the response of tissues to folate antagonists in general. Likewise, dietary FA could be an effective means to regulate plasma and tissue folates. There have been a number of studies in animals [14, 15] and humans [16] demonstrating that dietary FA deficiency leads to depletion of reduced folates. Recently, it was shown that intentional dietary FA depletion caused mice to become much more sensitive to the toxic effects of DDATHF and more like humans in this regard [7]. Further, modest repletion of mouse dietary FA was shown to result in alleviation of toxicity without extensive loss of antitumor activity. This type of modulation by dietary FA could lead to improved therapeutic use of DDATHF, and other antifolates, provided it is properly understood. One critical aspect of this understanding is the behavior of reduced folate pools in response to dietary FA changes in specific tissues, and their relationship to plasma.

To fully define the response of tissues to dietary FA changes, it is necessary to evaluate all reduced folates, so that individual as well as total folate behavior can be considered. The ternary complex assay system employed in this study permitted estimation of each naturally occurring folate at a radioisotopic level of sensitivity without the necessity of radiolabeled folate administration [13]. Even low levels of reduced folates in animals on FA deplete diets could be readily detected with this approach.

When folates were estimated by the ternary

complex assay, there was a 6-fold depletion of total folate in plasma of mice placed on an FA deplete diet but only a 50% reduction of levels in RBCs, intestine, and liver (see Table 1). RBCs were investigated because of their potential as a readily available tissue sensitive to systemic folate deficiency. RBCs are thought to acquire all of their folate content during development with essentially no accumulation as mature erythrocytes [17] due to a loss of FPGS [18]. On the other hand, liver and other tissue that reportedly contain relatively high FPGS activity [19] can acquire folate on a relatively constant basis to survive. This would mean that RBC folate levels would depend upon both the time and degree of systemic depletion while other tissues depend on plasma levels alone. Nevertheless, the fractional loss of total folate in mice on an FA deplete diet was nearly the same in RBCs as in liver and intestinal tissue. Thus, if this observation can be extended to shorter and longer periods of deficiency, RBCs may satisfy the criteria for a readily available source of tissue to evaluate folate depletion during dietary deficiency.

Results shown in Tables 1 and 3 indicate that total folates in normal mouse tissues (liver and intestine) were diminished by about 50% in response to a 2-week dietary FA depletion. On the other hand, tumor tissue responded much more dramatically (see Table 4). The response in tumor was almost as great as that in plasma (see Tables 1 and 2). Because tumor responds more profoundly, it could be more susceptible to dietary modulation than normal tissue, providing a more sensitive system for antifolate targeting.

Administration of DDATHF 1 week prior to examination of tissues had little effect on reduced folates in mice on either FA deplete or replete diets. Further, short-term (2-day) administration of FA or the reduced folate 5-$CHOFH_4$ elevated total folates to about the same extent. However, it should be pointed out that in plasma of FA-treated animals, the total folate content was apparently made up substantially of FA itself, whereas in the case of 5-$CHOFH_4$ none of the parent compound was present. Although FA is reported as a combined pool with $FH_2$, it is very unlikely that the latter folate makes a significant contribution. Failure to observe significant levels of circulating [*S*]5-$CHOFH_4$ following oral administration is consistent with previously reported results in humans [13, 20, 21]. Not only was the relative elevation of reduced folate pools the same for FA and 5-$CHOFH_4$, FA at three times the level used to compare with 5-$CHOFH_4$ caused essentially the same elevation. These results suggest that substantial saturation occurs below the lowest level of either folate. This saturation is presumably at the level of tissue uptake rate, because it is clearly possible to attain higher folate levels in FA replete animals.

10-$CHOFH_4$, because it is a substrate for the DDATHF target enzyme GAR transformylase, would be anticipated to be the reduced folate most directly involved in tissue sensitivity to the drug [3]. It is interesting that tumor tissue in both control and folate-deficient animals was devoid of detectable 10-$CHOFH_4$ (see Table 4). Yet this pool was elevated substantially in response to both FA levels and 5-$CHOFH_4$. This is presumably the result of a strong DDATHF block of GAR transformylase. Thus, the relative content of this reduced folate and accumulation of DDATHF in tumor tissue in the *in vivo* setting may make a significant contribution to the antitumor activity of DDATHF. This possibility will be investigated more extensively in the future.

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