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Impact of Schedule on Leucovorin Potentiation of Fluorouracil Antitumor Activity in Dietary Folic Acid Deplete Mice

*Kamasamudram Raghunathan, John C. Schmitz and David G. Priest**

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY, MEDICAL UNIVERSITY OF SOUTH CAROLINA, CHARLESTON, SC 29425, U.S.A.

ABSTRACT. A dietary folic acid depleted mouse model was established and used to evaluate the relationship between elevation of reduced folates after leucovorin (LV) administration and potentiation of fluorouracil (FU) response of an implanted tumor. C3H mouse mammary adenocarcinomas from mice maintained on a folic acid deplete diet had modestly decreased methylenetetrahydrofolate and tetrahydrofolate levels, and were somewhat less responsive to FU alone compared with replete animals. LV administration resulted in a substantial increase in tumor folate by 1 hr that returned to near basal levels by 12 hr. Reduced folates were elevated to a lesser extent in animals on a standard diet. Tumor growth was suppressed approximately 80% when FU was administered to depleted animals 1 hr after LV administration, compared with approximately 50% suppression in control mice. LV administered 12 hr before FU resulted in tumor growth stimulation that was consistent with the pronounced growth stimulation when LV was administered without FU. These results show that dietary folic acid depletion can lead to a more responsive FU/LV model and that administration of LV at an improper time before FU not only can fail to potentiate but also can result in tumor growth stimulation. BIOCHEM PHARMACOL 53;8:1197–1202, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dietary folate depletion; leucovorin; fluorouracil; mammary adenocarcinoma; mouse model

LV† in combination with FU has become an important therapy for the treatment of advanced colorectal and other types of cancer [1–3]. *In vitro* studies have shown that elevation of the LV metabolite, CH_2FH_4, stabilizes an inhibitory ternary complex formed between TS and the active metabolite of FU, FdUMP [4, 5]. In turn, depletion of thymidylate leads to diminished DNA synthesis and repair [6]. While it is clear that elevation of tumor CH_2FH_4 after LV administration causes the observed clinical response, the impact of the time interval between LV and FU administration, to allow metabolite accumulation, is poorly understood.

Because it is impractical to sample human tumor tissue sufficiently to monitor LV metabolite elevation [3, 7, 8], animal models have been sought. Unfortunately, only modest potentiation by LV has been reported in mice even with tumors that respond substantially to FU alone [9–12]. It has been suggested that elevated thymidine could play a role in response failure [12], but it is also possible that this lack of responsiveness is associated with the relatively high level of folic acid typically found in laboratory diets [13]. This folic acid enrichment could elevate basal tissue folates to an extent that little further elevation is possible, or it could cause other cellular changes that limit the capacity of LV to modulate FU activity [14]. Hence, a study was undertaken in mice with an implanted mammary tumor which were maintained on a low folic acid diet to investigate the role of the time interval between LV and FU administration on responsiveness.

MATERIALS AND METHODS

Materials

LV was obtained from Ben Venue Laboratories (Bedford, OH). C3H mammary adenocarcinoma was obtained from Lilly Research Laboratories (Indianapolis, IN) and was maintained in C3H mice (Charles River Co., Wilmington, MA) as s.c. axillary implants with a passage time between 14 and 20 days. Folic acid deplete chow (No. 5831C-2) containing 0.01 ppm folic acid and 1% succinylsulfathiazole to deplete intestinal flora, and control chow (No. 5001) containing 5.9 ppm folic acid, were purchased from Purina Mills (Richmond, IN). [^{3}H]FdUMP was purchased from Moravek Biochemicals (Brea, CA). Sephadex G-25 was obtained from Pharmacia (Piscataway, NJ). NADPH,

* Corresponding author: David G. Priest, Ph.D., Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425. Tel. (803) 792-4321; FAX (803) 792-4322.

† *Abbreviations:* LV, leucovorin; FU, fluorouracil; CH_2FH_4, 5,10-methylenetetrahydrofolate; FH_4, tetrahydrofolate; 5-CH_3FH_4, 5-methyltetrahydrofolate; 10-$CHOFH_4$, 10-formyltetrahydrofolate; TS, thymidylate synthase; and FdUMP, fluorodeoxyuridine monophosphate.

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ATP and all other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO). TS (4 U/mg protein) was purified from an *Escherichia coli* strain that overproduces *Lactobacillus casei* TS [15]. The *E. coli* strain was a gift from D. Santi (University of California, San Francisco). 5,10-Methylenetetrahydrofolate reductase (0.52 U/mg protein) and 10-formyltetrahydrofolate dehydrogenase (0.2 U/mg protein) were purified from pig liver as described previously [16, 17].

Animal Experiments

Mice that were maintained on the folic acid deplete diet were monitored for weight gain or loss and found to be no different from the control group (average weight = 22 g). Mouse mammary adenocarcinoma was excised from seed mice, cut into pieces small enough to fit a 13-gauge trocar, and implanted s.c. LV and FU were diluted in sterile saline and injected i.p. in a total volume of 0.3 mL/mouse. Tumor size was evaluated with graduated calipers, and volume was calculated as length × width × height × 0.5 [10]. Mice were killed in a CO_2 chamber to obtain blood, liver, and tumor samples.

Tissue Preparation

Whole blood (~600 μL) was collected by cardiac puncture and centrifuged immediately at 400 *g* for 5 min. The plasma obtained was diluted with an equal volume of cold 50 mM Tris–HCl buffer (pH 7.4) containing 50 mM sodium ascorbate and 1 mM EDTA and stored at −70°. Liver and tumor were excised from mice, washed with cold PBS, and stored at −70°. For folate analysis, tissues were homogenized in cold 50 mM Tris-HCl buffer (pH 7.4) containing 50 mM sodium ascorbate and 1 mM EDTA, and centrifuged at 10,000 *g* for 10 min at 4° to remove cell debris. An aliquot of the supernatant was used for soluble protein determination by the method of Bradford [18]. The remainder of the supernatant was placed immediately in a boiling water bath for 5 min and centrifuged to remove precipitated protein. The resultant supernatants were used for folate estimation. Because CH_2FH_4 can potentially be dissociated to FH_4 and formaldehyde under these conditions, the sum of these folates is reported. Other reference folates were stable under these conditions, with routine recovery in the range of 70–95% [19].

Estimation of Reduced 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 *L. casei* TS and [^{3}H]FdUMP. Methods have been described previously for estimation of the biologically active stereoisomers of CH_2FH_4, FH_4, 5-CH_3FH_4, and 10-$CHOFH_4$ using this approach [13]. Typically, reaction mixtures contained 20 mU TS and 125 nM [^{3}H]FdUMP (20 Ci/mmol) in 200 μL of 50 mM Tris–HCl buffer (pH 7.4) containing 50 mM sodium ascorbate and 1 mM EDTA. Additional enzymes and cofactors were added as necessary to cycle each reduced folate to the CH_2FH_4 form. Ternary 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 applied to Sephadex G-25 mini columns and eluted 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 [19].

Estimation of Folate Polyglutamate Chain Length

The polyglutamate chain length of the tumor CH_2FH_4 + FH_4 pool was determined by electrophoresis of undenatured ternary complexes followed by fluorography [20]. Tumors were homogenized in 50 mM Tris-HCl buffer containing 50 mM sodium ascorbate, 1 mM EDTA, and 0.01 N NaOH to protect against enzymatic hydrolysis of polyglutamates during extraction. After 5 min at 4°, the pH was lowered to 7.4 with 1 N HCl, and precipitated protein was removed by centrifugation. Tumor supernatants were incubated with 125 nM [^{3}H]FdUMP, 6.5 mM formaldehyde, and 20 mU TS for 30 min before electrophoresis.

Statistical Analysis

All results are reported in terms of the numerical mean with the associated SEM. Confidence levels for differences between values are based on Student's *t*-test.

RESULTS

To determine the impact of dietary folic acid on plasma and liver reduced folates, mice were placed on a diet of laboratory chow that did not include the typical folic acid supplementation (0.01 vs 5.9 ppm folic acid). The ternary complex assay was used to monitor all natural reduced folates over an 8-week period. It can be seen in Fig. 1 that total plasma folate, based on summation of individual folates, decreased in a biphasic pattern. There was a 7-fold decrease during the initial phase with a half-life ($T_{1/2}$) of approximately 2 days. This phase lasted 9 days. Loss during the second phase was much slower with a $T_{1/2}$ of over 25 days. Total folates in liver were also depleted in a biphasic manner (Fig. 1). However, the $T_{1/2}$ for the initial phase was considerably longer than for plasma (21 days), and the second phase began after only a 60% decrease. Although results are not shown for individual folates, plasma folate consisted almost entirely of 5-CH_3FH_4 while liver contained substantial amounts of both 5-CH_3FH_4 and CH_2FH_4 + FH_4, with nearly twice as much of the latter. 10-$CHOFH_4$ was present in liver initially but represented less than 10% of the total pool and diminished to undetectable levels after 9 days. As folate depletion proceeded, the relative CH_2FH_4 + FH_4 content in liver fell more rapidly than

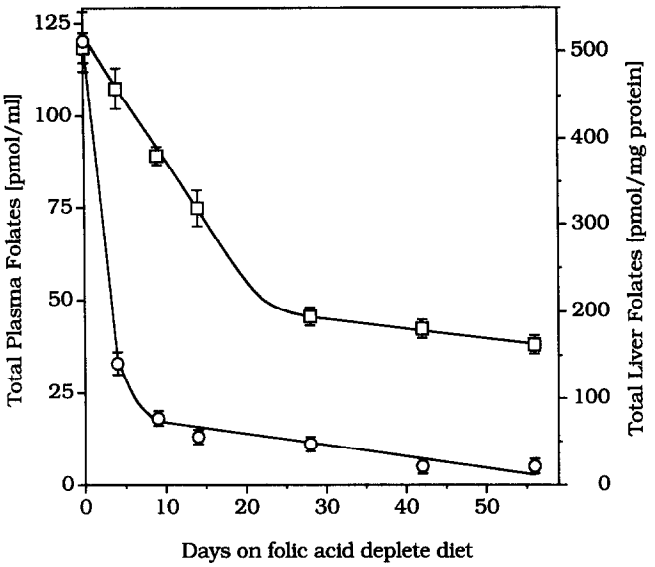

FIG. 1. Effect of dietary folic acid depletion on total reduced folates in mouse plasma and liver. Reduced folates from plasma (○) and liver (□) of mice maintained on a folic acid deplete diet were estimated by the ternary complex assay and summed to obtain total folates. Folate estimates represent the mean ± SEM of duplicate analyses of tissue from six mice.

the 5-CH_3FH_4 pool so that by 8 weeks these pools were almost equal.

These results indicated that a near constant and minimal folate content could be achieved in plasma after 2 weeks on the folic acid deplete diet. While this appeared to be an appropriate time for tumor implantation and drug evaluation, the fact that liver folates had not as yet achieved an equally constant level at this time was of concern. Hence, many of the experiments to be described subsequently were also conducted after 4 weeks on the deplete diet when liver folate had also reached a relative plateau, with no significant difference in response.

After 2 (or 4) weeks of maintenance on the folic acid deplete diet, mammary adenocarcinoma was implanted s.c. and allowed to grow for 7 days. The change in tumor size over the next 10-day period was used to assess treatment impact. Table 1 shows that the folic acid deplete diet alone caused a modest suppression of tumor growth compared with that of animals on a standard diet ($P = 0.09$). When FU at a dose of 10 mg/kg was administered every other day, tumor growth was suppressed in both groups but to a lesser extent in folic acid deplete animals than in those receiving a standard diet ($P = 0.09$). Tumor growth suppression was more extensive for a 25 mg/kg FU dose, and the difference between deplete and replete diets was more pronounced ($P = 0.004$).

Because it was anticipated that tumor accumulation of the LV metabolite, CH_2FH_4, would be the primary determinant of FU modulation, this pool, along with the closely related FH_4 pool, was measured. It can be seen in Table 2 that there was only a modest difference between basal levels of this folate pool in deplete versus replete animals ($P = 0.20$). One hour following LV administration (90 mg/kg), the $CH_2FH_4 + FH_4$ pool was elevated in animals on both diets, but the elevation was substantially greater in the deplete group. This was due primarily to a greater capacity for elevation in deplete animals. The final level achieved was considerably greater for the deplete versus the replete group ($P = 0.04$). By 12 hr, the $CH_2FH_4 + FH_4$ pool had returned to near basal levels in both groups.

TABLE 1. Effect of dietary folic acid on tumor growth response to FU

FU (mg/kg)	Tumor growth (mm^3)	
	Dietary folic acid	
	Deplete	Replete
0	942 ± 123	1194 ± 152
10	784 ± 36	874 ± 42
25	411 ± 48	179 ± 34

Control animals and mice maintained on a folic acid deplete diet for 2 weeks were implanted s.c. with a mammary tumor. After 1 week, animals were injected i.p. with 0, 10, or 25 mg/kg FU every other day for 9 days. On day 10, tumor volume was estimated and subtracted from estimates at the initiation of drug treatment. Values are means ± SEM from five mice.

Polyglutamylation of folate pools is an additional metabolic transformation that could potentially have an impact on modulation. Hence, polyglutamate chain length was evaluated by electrophoretic separation of ternary complexes formed from tumor folates of animals on the folic acid deplete diet [20]. It can be seen in Fig. 2 (lane 2), that the polyglutamate chain length distribution before LV administration was centered around 6 and 7 glutamate residues. However, 1 hr after LV administration (lane 3), only monoglutamate and a small amount of diglutamate were present. It should be pointed out that while an approximately equal quantity of folate-containing complex was applied to each lane, quantitative comparison of polyglutamate content cannot be made between lanes. The band intensity in lane 3 was expected to result mostly from "new" folate because the pool had been elevated substantially, but the preexisting polyglutamylated pool could rep-

TABLE 2. Elevation of tumor $CH_2FH_4 + FH_4$ following LV administration

Time (hr)	Tumor $CH_2FH_4 + FH_4$ (pmol/mg protein)	
	Dietary folic acid	
	Deplete	Replete
0	16 ± 6	23 ± 3
1	116 ± 6	73 ± 2
12	26 ± 5	23 ± 4

Mammary tumors were implanted s.c. into control animals, and mice were maintained on a folic acid deplete diet for 2 weeks. Ten days later, LV (90 mg/kg) was administered i.p. Tumors were excised 1 and 12 hr later, and $CH_2FH_4 + FH_4$ was determined by the ternary complex assay. Folate values are means ± SEM from duplicate analyses of tumors from four mice.

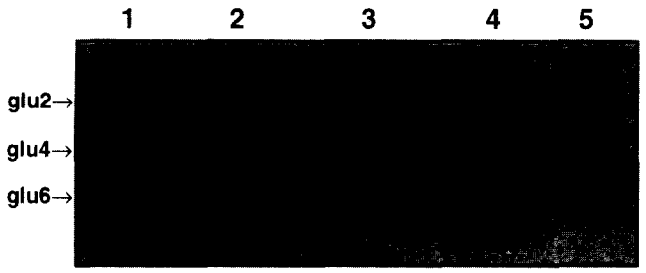

FIG. 2. Change in polyglutamate status of the CH_2FH_4 + FH_4 pool in mouse tumor 1 and 12 hr after LV administration. Reduced folates were extracted from tumors of folic acid deplete mice at 0 (lane 2), 1 hr (lane 3), and 12 hr (lane 4) after i.p. administration of 90 mg/kg LV. Polyglutamate chain length was estimated by the ternary complex method. Lanes 1 and 5 contain reference complexes with 2, 4, and 6 glutamate residues.

resent as much as 15% of the total if it remained. However, no bands associated with longer chain length polyglutamates were observed, even though they would be readily detectable at this level. The early propensity for monoglutamate was reversed completely 12 hr after LV administration (lane 4). The pool at this time consisted exclusively of folate with 5 and 6 glutamate residues.

The impact of dietary folic acid on LV modulation of FU antitumor activity is shown in Table 3. It can be seen that when LV (90 mg/kg) was administered daily to folic acid deplete mice 1 hr before FU (10 mg/kg), tumor growth was suppressed approximately 80% compared with untreated controls. On the other hand, growth was suppressed only about 50% in animals maintained on the standard high folic acid diet. Of greater interest with regard to establishment of a LV modulation model was the growth suppression by nearly 75%, compared with FU alone, in animals on the deplete diet, while tumors from replete animals responded only modestly (P = 0.001). Interestingly, this modulation effect in deplete animals was not observed when FU was administered 12 hr after LV. Instead, there was a 30% increase in growth compared with untreated controls, and tumor size was almost doubled compared with FU alone. By 12 hr, the CH_2FH_4 + FH_4 pool had returned to near basal levels. This tumor growth stimulation effect most likely results from failure of the modestly elevated reduced folate pool at this time to overcome the strong stimulation seen when LV was administered alone (Table 3). In this case, tumor growth more than doubled in folic acid deplete animals and even increased over 90% in the replete group.

TABLE 3. Impact of time interval between LV and FU administration on tumor growth

LV (mg/kg)	FU (mg/kg)	Time interval (hr)	Tumor growth (mm^3) Dietary folic acid: Deplete	Replete
0	0		942 ± 123*	1194 ± 152*
90	10	1	194 ± 17	612 ± 71
0	10		761 ± 28	814 ± 63
90	10	12	1240 ± 160	ND†
90	0		2014 ± 405	2295 ± 568

Mammary tumors were implanted s.c. into control animals, and mice were maintained on a folic acid deplete diet for 2 weeks. One week later, FU and/or LV was administered i.p. daily for 9 days. On day 10, tumor volume was estimated and subtracted from estimates at the initiation of drug treatment. Values are means ± SEM from five mice.

* Results from Table 1 for comparison.

† ND = not determined.

DISCUSSION

Because tumor elevation of the active LV metabolite, CH_2FH_4, is required for modulation of FU activity, the time interval between their administration is expected to be a determinant of response. However, establishment of the optimal interval requires detailed evaluation of tissue folates in conjunction with antitumor response. An animal model is the only practical way to obtain such results. The mouse model used for this study, which was based on restriction of dietary folic acid, was clearly far more responsive to LV modulation than mice maintained on standard, high folic acid diets. It should be pointed out that this system also yielded plasma folate levels that were more comparable to the approximately 10 nM level typically seen in human plasma [19]. Mice maintained on a standard diet had plasma folate levels that were approximately 120 nM, while the deplete diet led to levels of 10 nM and below (see Fig. 1).

The original hypothesis behind this study was that standard diets, enriched in folic acid, result in tissue folate levels that are too high for substantial further elevation by LV. Interestingly, the greater elevation of folate in tumors from animals on the deplete diet compared with replete animals did not result as much from lower basal levels before LV administration as from the propensity to achieve higher levels in the deplete group. The reason for this higher capacity to accumulate folate might be associated with more avid uptake. Higher levels of transport and/or binding proteins have been observed previously in response to folic acid depletion [14].

There have been a wide range of schedules used clinically to administer FU and LV [21–23], but it has been difficult to associate a precise degree of response to schedule in patient populations. This can be attributed in large part to a lack of knowledge about tumor tissue elevation of active modulatory metabolites. FU has a particularly short half-life [24] which would tend to make the time at which it is administered especially critical. In this model system, tumor folates were elevated substantially 1 hr following LV administration.‡ Likewise, excellent antitumor activity was achieved when FU was administered at this time. However, 12 hr after LV administration, when folates had returned to near basal levels, FU administration not only failed to sup-

press growth but modest growth stimulation was observed. This stimulation can likely be attributed to folate elevation during a period when no FU is present. That LV can stimulate growth was demonstrated in this study by administration of LV alone (see Table 3), and has been observed by others [11]. This report should raise concern that when FU is administered in conjunction with LV, therapeutic failure, or even tumor growth stimulation, may occur if a proper interval between LV and FU is not considered. It has been observed, using this same model system, that maximal tumor elevation of the CH_2FH_4 + FH_4 folate pool is coincident with maximal elevation in plasma.‡ Hence, it is proposed that consideration of human plasma elevation of these reduced folates [19] could be used as a basis to establish the optimal time for clinical administration of FU after LV.

It has been shown that polyglutamylation of CH_2FH_4 leads to more avid stabilization of the inhibibitory TS ternary complex [25]. However, it has not been clearly established that polyglutamylation is essential for effective modulation therapy with FU/LV. It is possible that sufficient tumor elevation of CH_2FH_4, as the monoglutamate, could have the same inhibitory potential as lesser elevation of polyglutamylated folate. Results reported here support this latter concept. Excellent antitumor activity was obtained when FU was administered 1 hr after LV, at which time essentially the entire tumor reduced folate pool was in the monoglutamate state. Alternatively, at 12 hr post LV, when the modestly elevated CH_2FH_4 + FH_4 pool had been fully polyglutamylated, no antitumor activity was seen. Admittedly, this conclusion is clouded somewhat by the growth stimulatory effects of folate during periods when FU was not present, but the near qualitative difference in response strongly suggests that it is at least possible to achieve substantial modulation with sufficiently high levels of folate monoglutamates.

Failure to observe any evidence for longer chain length polyglutamates in samples taken 1 hr after LV administration was unexpected because at least 15% of the CH_2FH_4 + FH_4 pool present at that time could have been residual folate polyglutamates. This loss of the preexisting pool by 1 hr suggests that a facile pathway exists to cycle cellular folate polyglutamates in this system. Such a facile pathway would presumably involve folylpolyglutamate hydrolase. In turn, hydrolytic cleavage has been reported to occur both exo- and endolytically [26, 27]. In this case, an endolytic process appears most likely because of the absence of intermediate chain length species. However, earlier evaluation of folates would be necessary to be certain that intermediates have not already been completely cleaved back to the monoglutamate state.

In summary, an improved mouse model for FU/LV evaluation was achieved by limiting dietary folic acid. This model was used to show that the antitumor activity that can be attained if FU is administered at a time when tumor folates are elevated, is not only reversed, but tumor growth stimulation can occur when FU is administered at times when folates are not elevated sufficiently. In addition, this murine model suggests that polyglutamylation of the elevated folate pool is not essential for achievement of excellent response. Future investigations with this system will center upon establishment of the precise degree to which tumor folate must be elevated to achieve maximal antitumor activity.

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