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Disposition of leucovorin and its metabolites in the plasma, intestinal epithelium, and intraperitoneal L1210 cells of methotrexate-pretreated mice

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Abstract. Leucovorin (LV or 5-$CHOFH_4$) has had long-standing clinical use as a rescue agent from the systemic toxic effects of methotrexate (MTX). Because the mouse has been the animal model most used to investigate MTX therapy, direct tissue assessment of LV and its reduced-folate metabolites was undertaken in the plasma, intestinal epithelium, and intraperitoneal L1210 cells of MTX-pretreated mice using a ternary-complex-based assay method. The results show that total folate accumulation and depletion in tissues is closely related to plasma levels, with somewhat greater persistence occurring in tissues, presumably due to polyglutamylation. Examination of individual folates in plasma showed that the combined 5,10-methylenetetrahydrofolate (CH_2FH_4) plus tetrahydrofolate (FH_4) pool was the most extensively elevated pool other than that of the parent compound [S]-5-formyltetrahydrofolate ([S]-5-$CHOFH_4$). The dihydrofolate (FH_2) also became elevated, whereas the 5-methyltetrahydrofolate (5-CH_3FH_4) remained unchanged. Individual folates that were elevated in tissues were generally the same as those elevated in plasma, the exception being a significant accumulation of 10-formyltetrahydrofolate (10-$CHOFH_4$) in both intestinal epithelial and L1210 cells. The elevation of FH_2 in L1210 cells was greater and persisted longer than that in intestinal epithelium, whereas the opposite was true for CH_2FH_4 + FH_4. This differential effect in tumor versus epithelial tissue is consistent with the selective rescue of normal tissue by LV.

Key words: Leucovorin – Methotrexate – Folates – L1210 murine leukemia

Introduction

The mouse has been the animal model used most to investigate leucovorin (LV or 5-$CHOFH_4$) rescue from toxic doses of methotrexate (MTX) [1, 2]. However, direct evaluation of the behavior of LV and its active reduced-folate metabolites has been limited. Only the parent compound and 5-methyltetrahydrofolate (5-CH_3FH_4) have been estimated in the plasma of mice [3, 4], yet other reduced folates have been observed in human plasma following LV administration [5]. Furthermore, tissue studies have been restricted to liver, bone marrow, and tumor [2–4, 6] and have not included evaluation of all natural reduced folates such that the level of each folate could be considered relative to the total. In addition, most folate studies in mice have been restricted to very high doses of LV and have not considered animals treated with MTX.

Generally, comprehensive in vivo studies of plasma and tissue folates have been limited by the analytical methods available. Low levels of unstable metabolites are difficult to detect using classic analytical methods without prior uniform radioisotopic labeling of all folate pools [7], a task that is impractical in animals and humans. A method based on enzymatic cycling of folates to 5,10-methylenetetrahydrofolate (CH_2FH_4) followed by entrapment into a stable ternary complex with thymidylate synthase, labeled by the secondary ligand [^{3}H]-5-fluoro-2′-deoxyuridine-5′-monophosphate ([^{3}H]-FdUMP), has now been expanded to include all of the natural reduced folates [5, 8, 9]. This method was used to estimate levels of LV and its metabolites in the plasma, intestinal epithelium (the site of MTX dose-limiting toxicity), and intraperitoneal L1210 cells (a murine leukemia tumor cell line) of mice pretreated with MTX.

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Abbreviations: MTX, methotrexate; LV or 5-$CHOFH_4$, leucovorin or 5-formyltetrahydrofolate; FU, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FH_2, dihydrofolate; FH_4, tetrahydrofolate; CH_2FH_4, 5,10-methylenetetrahydrofolate; 5-CH_3FH_4, 5-methyltetrahydrofolate; 10-$CHOFH_4$, 10-formyltetrahydrofolate

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Materials and methods

Drugs. [6-^{3}H]-FdUMP (20 Ci/mmol) and [3′,5′,7,9-^{3}H]-[6S]-5-$CHOFH_4$ (40 Ci/mmol) were purchased from Moravek Biochemicals (Brea, Calif.). [^{3}H]-5-$CHOFH_4$ was routinely purified by high-performance liquid chromatography (HPLC) prior to its use as previously described [10]. Unlabeled [6R,S]-5-$CHOFH_4$ was obtained from the Drug Development Branch, National Cancer Institute (Bethesda, Md.). MTX was purchased from Lederle Laboratories (Pearl River, N.Y.) and Sephadex G-25 was obtained from Pharmacia (Piscataway, N.J.). Reduced nicotinamide adenine dinucleotide phosphate (NADPH), adenine 5′-triphosphate (ATP), and all other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.). Thymidylate synthase and other enzymes used to estimate reduced folates by the ternary-complex assay were purified as previously described [5, 8, 9].

Tumor transplantation and drug administration. Transplantation of L1210 murine leukemia cells into female C57Bl × DBA/2 Fl mice was conducted as described previously [2]. Mice were injected intraperitoneally with 10^4 cells. When the cell density reached $100-200\times10^6$ cells/animal, MTX was injected subcutaneously at a dose of 400 mg/kg. LV was injected subcutaneously 16 h following MTX administration.

Preparation of tissue samples. L1210 cells, collected from two animals for each time point, were harvested from the peritoneal cavity, combined, and washed once with cold 0.175 *M* NH_4Cl and once with cold, buffered 0.14 *M* NaCl/0.01 *M* sodium phosphate (pH 7.4). The combined cells were resuspended in a buffer containing 50 m*M* TRIS-HCl, 50 m*M* sodium ascorbate, and 1 m*M* ethylenediaminetetraacetic acid (EDTA, pH 7.4) at a density of 20×10^6 cells/ml and then placed in a boiling water bath for 3 min to achieve lysis. Extracts were centrifuged at 15,000 *g* for 5 min at 4° C. Intestines from two animals were collected for each time point and the proliferative fractions were isolated by stripping the nonproliferative fraction from the everted small intestine by agitation for 12 min in 0.14 *M* NaCl/0.01 *M* sodium phosphate (pH 7.4) at 4° C. The proliferative fractions from two animals for each time point were then scraped free, combined, and used for analysis.

Extracts of proliferative epithelial cells were prepared by homogenization of approximately 0.1 g tissue in 20 ml 50 m*M* TRIS-HCl buffer containing 50 m*M* sodium ascorbate and 1 m*M* EDTA (pH 7.4) followed by centrifugation at 15,000 *g* for 15 min at 4° C. Supernatants were boiled for 3 min to denature undesirable enzymes and then recentrifuged.

Blood samples, obtained from two animals for each time point, were collected from the orbital sinus and combined. Plasma was rapidly prepared by centrifugation of whole blood at 4,000 *g* for 5 min at 4° C and was diluted into an equal volume of 50 m*M* TRIS-HCl buffer containing 100 m*M* sodium ascorbate (pH 7.4). Plasma was then deproteinated by boiling for 3 min followed by centrifugation at 15,000 *g* for 5 min at 4° C.

Estimation of reduced folates. Tissue extracts and plasma samples (30–80 µl) were used to estimate the following tissue levels of reduced folates as described previously: CH_2FH_4, tetrahydrofolate (FH_4), dihydrofolate (FH_2), 5-CH_3FH_4, 10-formyltetrahydrofolate (10-$CHOFH_4$), and [S]-5-$CHOFH_4$ [5, 9]. Because of concern regarding the interconversion of CH_2FH_4 and FH_4 during the boiling step of sample preparation [5], the sum of CH_2FH_4 and FH_4 is reported. Protein concentration was determined by the method of Bradford [11].

Estimation of total folate content by radioisotope accumulation. [^{3}H]-5-$CHOFH_4$ was used to estimate total folate accumulation in intraperitoneal L1210 cells. In brief, 10 µCi [3′,5′,7,9-^{3}H]-[6S]-5-$CHOFH_4$ (40 Ci/mmol) was injected subcutaneously into each of three mice at 16 h following MTX administration. L1210 cells were harvested approximately 20 min later, washed, and lysed as described above. Following centrifugation at 15,000 *g* for 5 min, the radioactivity content was estimated in supernatants by scintillation counting and then used to evaluate total folate elevation.

Results

A ternary-complex-based assay was used to investigate the behavior of subcutaneously injected LV and its metabolites in MTX-pretreated mouse plasma and tissues. Because all of the natural reduced folates can be detected with this approach, their summation permits estimation of the total folate pool. In turn, total folates can then be compared with the accumulation of radioisotopically labeled LV and its metabolites in tissue as an independent means of confirming the accuracy of the ternary-complex assay. When this comparison was made with L1210 cells isolated from animals that had received [^{3}H]-5-$CHOFH_4$ at a dose of 12 mg/kg, the total folate content was 178 ± 30 pmol/mg protein as determined by the radioisotopic method and 175 ± 10 pmol/mg protein as found by the ternary-complex method; those values are clearly in very good agreement. The parent compound 5-$CHOFH_4$ represented 20% of total folates as estimated by the ternary-complex assay.

To examine the time dependence of accumulation of LV and its metabolites in plasma and tissues, a 12 mg/kg dose

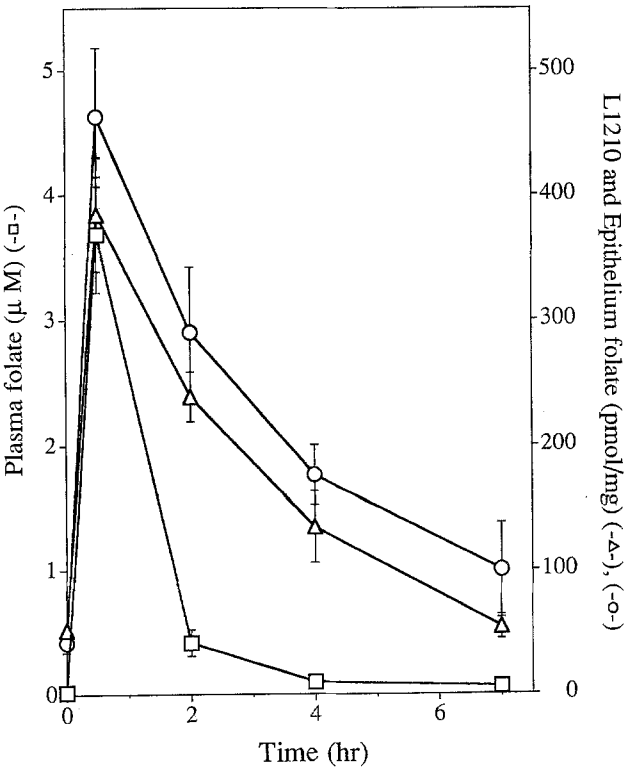

Fig. 1. Elevation of total reduced folates in MTX-pretreated mouse plasma (□), intestinal epithelial cells (○), and intraperitoneal L1210 cells (Δ) following subcutaneous LV administration at a dose of 12 mg/kg. Levels of each of the reduced folates 5-CH_3FH_4, CH_2FH_4, FH_4, FH_2, 10-$CHOFH_4$, and 5-$CHOFH_4$ were determined by the ternary-complex assay and summed to provide an estimate of total folate. Each data point represents the mean value ± SEM for 4 determinations conducted on pooled samples from 2 animals

Table 1. Distribution of individual reduced folates in MTX-pretreated mouse plasma, intestinal epithelial cells and L1210 cells following LV administration

Reduced folates	Time (h)	Plasma (n*M*)	Intestinal epithelial cells (pmol/mg)	L1210 cells (pmol/mg)
5-$CHOFH_4$	0	<1	<1	<1
	0.5	2,622±435	128±17	57±15
	2.0	133±57	57±23	8±4
CH_2FH_4 + FH_4	0	5±1	42±8	29±5
	0.5	876±169	123±41	138±28
	2.0	95±13	156±30	78±15
FH_2	0	1±0.7	<1	23±2
	0.5	137±100	57±9	71±11
	2.0	179±43	27±17	133±11
10-$CHOFH_4$	0	<1	<1	<1
	0.5	<1	117±30	119±31
	2.0	<1	6±5	19±6
5-CH_3FH_4	0	12±2	<1	<1
	0.5	13±4	38±10	<1
	2.0	12±8	44±31	<1

Mice were injected with 12 mg/kg LV at 16 h after the administration of 400 mg/kg MTX. Blood, intestines, and L1210 cells, collected from two animals for each time point, were combined and reduced folates were determined by the ternary-complex assay. Data represent mean values ± SEM for 4 determinations

was injected subcutaneously and levels of folates were monitored. It can be seen in Fig. 1 that total folates generally follow the same kinetic pattern in both intestinal epithelial and L1210 cells as in plasma. This correspondence is apparent not only for the accumulation phase but for depletion as well, although plasma folates returned to baseline levels, whereas tissue folates did not. Quantitatively, the total folate level achieved in tissues, based on 1 mg extracted protein being equivalent to 14 mg wet tissue weight, was approximately 5-fold that obtained in plasma.

Levels of individual reduced folates detected in each of the tissues and in plasma at 30 min and 2 h after LV administration are shown in Table 1. The lower total levels determined at later times generally exhibited the same pool distributions. Prior to administration, 5-CH_3FH_4 was the predominate plasma folate but, in contrast to the other folates, it was not elevated by LV in these MTX-treated mice. Plasma FH_2, which was near the detection limits prior to LV administration, became extensively elevated. Furthermore, in contrast to the other plasma folates, FH_2 remained at relatively high levels over the period examined. CH_2FH_4 + FH_4 was the pool most responsive to LV in plasma, increasing some 175-fold after 30 min but diminishing by 2 h. 10-$CHOFH_4$ was not observed in plasma prior to or after LV administration.

The kinetic pattern observed for the elevation and subsequent loss of individual folates after LV administration in the two tissues was much like the response seen in plasma. The CH_2FH_4 + FH_4 pool became extensively elevated in both intestinal epithelial and L1210 cells but was somewhat higher and more persistent in the normal tissue. On the other hand, although significant FH_2 accumulation also occurred in both tissues, it was generally higher and more persistent in the L1210 tumor cells. The parent compound 5-$CHOFH_4$ accumulated in both tissues but generally to a greater extent in the intestinal epithelium.

Discussion

LV is used therapeutically to rescue cancer patients from the toxic effects of MTX [12], but is has been difficult to evaluate the relationship between plasma and tissue folates following LV administration in humans undergoing MTX treatment. Although the mouse is not a perfect human model, it can be used to address specific questions regarding changes in folate pools. Following a bolus dose of LV, the kinetics of total folate elevation and depletion in mouse tissue is similar to that in plasma, as might be expected (see Fig. 1). Both tumor and intestinal epithelial cells accumulate reduced folates to a nearly 5-fold greater extent than is present in plasma. This observation contrasts with a previous report [4] in which the plasma levels of folates exceeded the concentrations in tissues. However, in that study, LV was given at a dose of 400 mg/kg as compared with the 12 mg/kg used in the present study. This difference is consistent with tissue folate saturation at very high doses, which suggests that administration of high single doses of LV to cancer patients for therapeutic purposes may be of limited value. The relatively small fraction of folate that is retained in tissues is presumably the result of polyglutamylation-dependent intracellular entrapment. This suggests that a longer-term infusion would be a preferable means of more permanently elevating tissue pools of reduced folate. It should be pointed out, however, that the amount of time for which folates must be elevated so as to reverse the toxic effects of MTX has been addressed only to a modest extent. If intracellular MTX displacement is an important factor, then reversal may be relatively rapid, depending more on the polyglutamate status of the antifolate than the duration of intracellular folate elevation.

The relatively high level of LV metabolites, namely the CH_2FH_4 + FH_4 and FH_2 pools, found in the circulatory system following LV administration is thought to result primarily from liver metabolism. The extent to which these one-carbon metabolites, as opposed to LV itself, contribute to reversal of MTX toxicity is currently not clear. However, their presence in plasma from systemic activation of LV makes most of the natural folates readily available to target tissues, regardless of the ability of these tissues themselves to metabolize LV. Polyglutamylation of folates, on the other hand, occurs only intracellularly and is thus expected to play an important role in folate retention only at the target site.

With regard to specific pools, 10-$CHOFH_4$ was not detected in plasma, whereas it was elevated in tissues (Table 1). Previous LV pharmacokinetics studies in human plasma [5] as well as recent studies in mice [13] also detected essentially no plasma level of 10-$CHOFH_4$. This folate is apparently readily converted to more reduced forms in the circulatory system. It has been shown in both

human and mouse plasma that 5-CH_3FH_4 is the predominant folate following LV administration, provided that no antifolate has been given [3, 5]. The failure to observe a significant elevation of this reduced folate in plasma from MTX-pretreated mice suggests that MTX interferes at some point in the metabolic pathway leading to the fully reduced form. However, the exact enzyme system(s) affected remains unknown.

The response of MTX-treated mice to LV administration in the present study was addressed under conditions (16 h after the administration of 400 mg/kg MTX) in which a substantial difference in MTX content between tumor and normal tissue has been observed [2]. In addition, previous studies showed that when LV (12 mg/kg) was given following MTX, a time-dependent loss of intracellular drug was induced, with a greater amount of MTX being lost from intestinal epithelial cells than from L1210 cells [2]. These earlier observations led to the suggestion that LV could be selective in the rescue of normal versus tumor tissue from MTX toxicity. The results of our examination of metabolite levels in intestinal epithelial and L1210 cells tend to support this concept. Even though total folate levels remained about the same, FH_2 was elevated to a greater extent and persisted longer in tumor cells as compared with intestinal epithelial cells, whereas $CH_2FH_4 + FH_4$ was more extensively elevated in intestinal epithelial cells and persisted for a longer period. Both of these metabolic consequences are consistent with superior rescue of intestinal epithelium as compared with L1210 cells. Furthermore, both could result from higher residual levels of MTX in L1210 cells [2]. The greater MTX content would tend to prevent utilization of reduced folates, causing more extensive elevation of FH_2 and depletion of $CH_2FH_4 + FH_4$. Future studies will address this question in more detail.

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