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Role of Folylpolyglutamates in Biochemical Modulation of Fluoropyrimidines by Leucovorin[1]

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ABSTRACT

The growth-inhibitory effect of fluoropyrimidines combined with a short-term exposure to leucovorin and the pattern of polyglutamylation of folates were compared between parental CCRF-CEM cells and a cell line with impaired ability to form polyglutamates (CCRF-CEM/P). The combination of leucovorin with 5-fluorouracil or 5-fluorodeoxyuridine increased the growth inhibition of CCRF-CEM cells compared to the fluoropyrimidine alone in the parent cell line but not in CCRF-CEM/P cells. In addition, leucovorin produced a significant increase in the inhibition of intracellular thymidylate synthase activity caused by 5-fluorouracil or 5-fluorodeoxyuridine as compared to these drugs alone in CCRF-CEM cells, but no increase in inhibition over that produced by the single drugs alone was observed in CCRF-CEM/P cells. Although levels of 5,10-methylene tetrahydrofolate after leucovorin administration were similar in both cell lines, polyglutamylation of this coenzyme was decreased in the CCRF-CEM/P cell line. The inability of CCRF-CEM/P cells to form significant levels of polyglutamates of N^5,N^{10}-methylenetetrahydrofolate, may be responsible for the lack of enhanced cell kill observed when a short exposure to leucovorin is used with fluoropyrimidines.

INTRODUCTION

One of the most commonly used regimens for the treatment of patients with metastatic colorectal cancer is 5-fluorouracil (FUra)[5] and high doses of leucovorin (LV) (1, 2). The rationale for this biochemical modulation is to provide the cells with large amounts of reduced folates, in an attempt to increase the intracellular levels of N^5,N^{10}-methylenetetrahydrofolate (CH_2FH_4) and its polyglutamate forms (3, 4). The affinity of CH_2FH_4, the cofactor required for the formation of the ternary complex with thymidylate synthase (TS) and fluorodeoxyuridylate (FdUMP) (5), increases with the number of glutamate residues associated with the folate (6). LV has been shown to increase the cytotoxicity of fluoropyrimidines in some cell lines (7, 8), and several randomized clinical trials have reported an increased rate of response with this combination over using FUra alone (9, 10). However, the majority of patients still do not respond to this regimen. Possible reasons of failure may be a lack of formation (11) or a deficiency in polyglutamylation of this folate cofactor (6). In the latter case, a short exposure to LV could fail to be effective because accumulated folates could rapidly efflux from the cells or could yield a less active cofactor for stabilization of the inhibitory complex (6). We compared the effect of the LV/fluoropyrimidine combination in CCRF-CEM (12) and in a methotrexate (MTX)-resistant subline of these cells, CCRF-CEM/P, which is impaired in its ability to polyglutamate MTX and folates (13, 14). As compared to the parental cells, the methotrexate-resistant cell line is less sensitive to the LV/fluoropyrimidine drug combination. Both CCRF-CEM/P and the parental line accumulate CH_2FH_4 in the presence of LV in a dose-dependent manner. However, the MTX-resistant cell line is less able to form longer chain length polyglutamates. The implications of this observation for clinical treatment is discussed.

MATERIALS AND METHODS

Chemicals. FUra and FdUrd were purchased from Sigma Chemical Co, St. Louis, MO. LV was obtained from Lederle, Pearl River, NY. 2′-[5-^{3}H]Deoxyuridine, (6*RS*)-[3′,5′,7′-^{3}H]LV and [6-^{3}H]FdUMP (20 Ci/mmol) were purchased from Moravek Biochemicals, Brea, CA, and the purity of the radiolabeled LV and FdUMP were confirmed by high-pressure liquid chromatography. An *Escherichia coli* strain that overproduces *Lactobacillus casei* TS was a gift from D. Santi, University of California, San Francisco, CA. TS was purified by a modification of the method of Pinter *et al.* (15). Folic acid polyglutamate standards with two to seven residues were obtained from C. M. Baugh, University of South Alabama, Mobile, AL, and were reduced to the corresponding tetrahydrofolates with *L. casei* dihydrofolate reductase and NADPH (16). Media, sera, and antibiotics for cell culture were obtained from Grand Island Biological Co., NY, and plastic ware was obtained from Corning Glass Works, Corning, NY.

Cell Lines. CCRF-CEM cells were from a cloned subline of the human T-lymphobast cell line described by Foley *et al.* (12). CCRF-CEM/P cells were obtained after selection with 3 μM MTX for seven cycles as described by Pizzorno *et al.* (13). These cells have a marked defect in their ability to form polyglutamates of MTX, and a moderate difference in their ability to form polyglutamates from folates, as a consequence of an alteration in the folylpolyglutamate synthetase enzyme (14). Cells were maintained as suspension cultures in RPMI 1640 medium (containing 2.3 μM folic acid) supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 atmosphere and subcultured twice a week. Under these conditions the doubling time of exponentially growing cells was 24 h. Both cell lines were periodically confirmed to be *Mycoplasma* free.

Cell Growth Inhibition Studies. Exponentially growing cells were exposed to drugs at an initial density of about 2×10^5 cells/ml. All drugs were dissolved in water and prepared freshly before each experiment. After drug treatment, cells were harvested by centrifugation, washed 3 times with phosphate-buffered saline, and resuspended in drug-free medium supplemented with 10% horse serum at a concentration of about 2×10^4 cells/ml. Subsequent changes in cell number were followed over a 5- to 6-day period by using a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

TS Activity. A modification of the *in situ* assay of Yalowich and Kalman (17) as described by Rodenhuis *et al.* (18) was used to measure TS activity. In brief, 2′-[5-^{3}H]deoxyuridine is converted to thymidylate, with release of the 5-^{3}H as ^3H_2O (16). TS activity was assayed at 0, 6,

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[5] The abbreviations used are: FUra, 5-fluorouracil; FdUrd, fluorodeoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; LV, leucovorin; TS, thymidylate synthase; MTX, methotrexate; FH_2, dihydrofolate; FH_4, tetrahydrofolate; CH_2FH_4, N^5, N^{10}-methylenetetrahydrofolate; 5-CH_3FH_4, 5-methyltetrahydrofolate; 10-$CHOFH_4$, 10-formyltetrahydrofolate.

24, and 48 h after exposure to the fluoropyrimidine with or without leucovorin. Cells were exposed to 2'-[5-^{3}H]deoxyuridine (2 μCi/ml) and at 0, 15, 30, and 45 min a 100-μl aliquot of cells was placed in 200 μl of 4% (w/w) trichloroacetic acid with 15% charcoal to stop the reaction. The mixture was centrifuged for 5 min and 100 μl of supernatant were pipeted into 5 ml of Ecolume (ICN) scintillation fluid and counted in a Beckman 5801 scintillation counter. A blank consisting of media without cells or drug was used for background subtraction. The counts were fitted to a straight line by linear regression and the percentage of inhibition was calculated by comparing the slope for treated cells with that for untreated cells.

LV Uptake. Exponentially growing cells (CCRF-CEM and CCRF-CEM/P) were harvested by centrifugation and suspended at a density of 1 to 3 × 10^7 cells/ml in complete medium in the presence of (6RS)-[3',5',7'-^{3}H]LV, generally labeled with a specific activity of 2–5 Ci/mmol, and unlabeled LV to a concentration of 10 μM. One hundred-μl aliquots were taken, in duplicate, at 5, 10, 15, 30, and 60 min and added to an ice-cold solution of 200 μl of 9% sucrose and 700 μl of 0.9% NaCl solution in microfuge tubes, centrifuged for 2 min at 16,000 × *g*, and washed twice with 1 ml of ice-cold 0.9% NaCl solution. The pellets were resuspended in 500 μl of 5% perchloric acid, boiled for 20 min, centrifuged for 2 min at 16,000 × *g*, and the entire supernatant was placed in Ecolume liquid scintillation fluid and counted in a Beckman Model 5801 scintillation counter.

Assay of Intracellular Folates. A series of radioenzymatic assays were used to measure FH_2, FH_4, CH_2FH_4, 5-CH_3FH_4, and 10-$CHOFH_4$ in cell-free extracts (19). These methods are based upon the entrapment of CH_2FH_4 by TS and [^{3}H]FdUMP to form a stable ternary complex (20). Cells were washed twice with cold PBS and suspended in extraction buffer that contained 50 mM Tris-HCl, 50 mM sodium ascorbate, 1 mM EDTA, and 0.25 M sucrose, pH 7.4. The final concentration of the cells was 2–3 × 10^7 cells/ml. Cells were boiled for 3 min to achieve lysis and to prevent enzymatic cycling during assay. The recovery of known amounts of CH_2FH_4 added to cell extracts prior to boiling was 94%.

Measurement of CH_2FH_4 and FH_4 Glutamate Chain Length. The polyglutamate chain length distribution of CH_2FH_4 and FH_4 was estimated by the electrophoretic separation of the ternary complex and fluorography of the label (21). Cells (2 × 10^7) were suspended in an extraction buffer as described above to give an intracellular CH_2FH_4 and FH_4 concentration of approximately 35 nM. Cells were lysed by freezing and thawing 3 times in dry ice/acetone in the presence of 125 nM [^{3}H]FdUMP and 19 milliunits *L. casei* TS, to prevent interference caused by hydrolysis of polyglutamates during sample preparation.

Statistical Analysis. To assess the significance of TS activity inhibition, four experimental conditions were compared: FUra *versus* FUra/LV in CCRF-CEM cells and in CCRF-CEM/P cells, and FdUrd *versus* FdUrd/LV in CCRF-CEM and CCRF-CEM/P cells. The square root transformation was used to stabilize the variance. The Greenhouse-Geiser adjustment to degrees of freedom was used in the *F* tests for within-subject effects. The Neuman-Keuls' method of multiple comparisons was also used (22). The *t* test for inequal variances was used when total folates were compared.

RESULTS

Tissue Culture Studies. When CCRF-CEM cells were exposed to LV (10 μM) for 4 h and to FUra (150 μM) during the last 2 h of LV exposure, potentiation of the inhibitory effect of FUra on cell growth was observed (Fig. 1). The addition of LV to FUra did not result in increased inhibition of cell growth in CCRF-CEM/P cells when compared to the cells treated with FUra alone (Fig. 1). Similar effects were observed when 100 μM LV was used (data not shown)

FdUrd (0.5 μM for 2 h) inhibited the growth of both cell lines. Pretreatment with 10 μM LV for 4 h starting 2 h prior to the exposure to FdUrd resulted in prolonged inhibition of cell growth in CCRF-CEM cells (Fig. 2). Potentiation was not

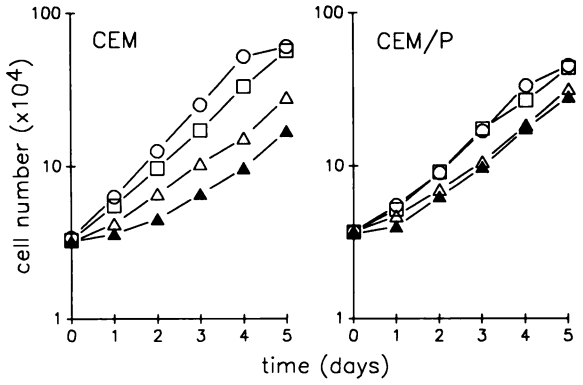

Fig. 1. Growth curves of CCRF-CEM and CCRF-CEM/P cells exposed to 10 μM LV for 4 h and 150 μM FUra during the last 2 h of LV exposure. Numbers represent the mean of 3–4 experiments. SE was less than 15% for each point. Control (○); LV (□); FUra (△); LV + FUra (▲).

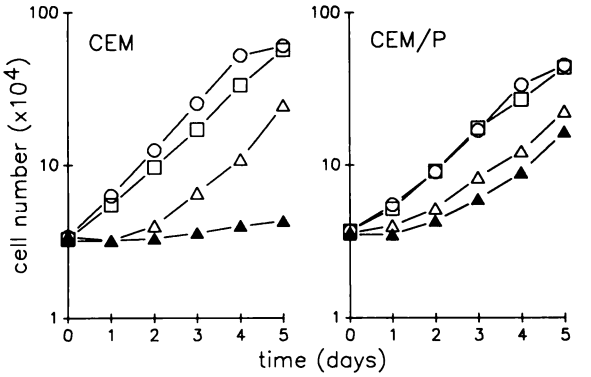

Fig. 2. Growth curves of CCRF-CEM and CCRF-CEM/P cells exposed to 10 μM LV for 4 h and 0.5 μM FdUrd during the last 2 h of LV exposure. Numbers represent the mean of 3–4 experiments. Standard error was less than 15% for each point. Control (○); LV (□); FdUrd (△); LV + FdUrd (▲).

observed in CCRF-CEM/P cells treated with FdUrd (0.5 μM) plus LV (10 μM) compared to the cells exposed to FdUrd alone (Fig. 2). Similar effects were seen when 100 μM LV was used (data not shown).

***In Situ* Thymidylate Synthase Activity Inhibition.** Cells (10^5 cells/ml) were exposed to 10 μM LV for 4 h and 150 μM FUra or 0.5 μM FdUrd alone for 2 h or during the last 2 h of LV exposure. The cells were washed free of drugs and LV. TS activity was determined immediately after washing (0 time) and after 6, 24, and 48 h of incubation in folate-free medium. In CCRF-CEM cells the addition of LV to FUra significantly increased the inhibition of TS activity at 0 and 6 h over drug alone ($P < 0.009$). In contrast, inhibition of TS activity was not significantly increased by one addition of LV to FUra over FUra alone in CCRF-CEM/P cells (Table 1; $P = 0.24$). After 24 and 48 h, there was no significant difference in the amount of TS activity inhibition with or without the addition of LV to FUra in either cell line (data not shown). The addition of 10 μM LV to FdUrd also significantly increased the inhibition of TS activity *versus* drug alone at 0 and 6 h in CCRF-CEM cells ($P < 0.01$), but not in CCRF-CEM/P cells ($P = 0.83$; Table 2). After 24 and 48 h, there continued to be a trend toward increased TS activity inhibition with the addition of LV to FdUrd in the CCRF-CEM line, but there was no difference in the CCRF-CEM/P line (data not shown).

LV Uptake. Uptake of 10 μM LV in CCRF-CEM and CCRF-CEM/P cells was similar, with total folate accumulation at 60

min of 22.6 ± 6.9 (SE) pmol/10^6 cells and 32.0 ± 4.8 pmol/10^6 cells, respectively (data not shown).

Estimation of Intracellular Folates. Prior to treatment, total reduced folates, based on the sum of CH_2FH_4, FH_4, FH_2, 10-$CHOFH_4$ and 5-CH_3FH_4 were 3.1 pmol/10^6 cells for both the parental and the MTX-resistant cell lines. When the parent or the CCRF-CEM/P cell line was exposed to 10 or 100 μM LV for 4 h, the total folates increased approximately 4- and 6-fold, respectively (Fig. 3*A*).

However, after a further 6-h incubation of cells in folate-free media to allow efflux of folates, the parental cells treated with 10 and 100 μM LV showed 2.8 and 3.1 times, respectively, more intracellular folates than the untreated control, whereas CCRF-CEM/P cells had only 1.6- and 1.3-fold increase in total folates *versus* the untreated control under the same conditions (Fig. 3*B*). The amount of total folates retained in CCRF-CEM *versus* CCRF-CEM/P cells exposed to 100 μM LV for 4 h and incubated in folate-free media for an additional 6 h was also significantly different ($P < 0.05$).

The levels of CH_2FH_4 increased to 5 and 11 times the control in CCRF-CEM cells exposed for 4 h to 10 and 100 μM LV, respectively, and increased to 5 and 8 times over base line in CCRF-CEM/P cells exposed for 4 h to 10 and 100 μM LV (Fig. 4*A*). After 6 h of efflux in folate-free medium, CH_2FH_4 levels decreased to 2.7 and 3 times higher than in the untreated control in CCRF-CEM cells exposed to 10 and 100 μM LV, respectively, and to 2 times higher than in untreated control in CCRF-CEM/P cells for both the LV doses (Fig. 4*B*).

Table 1 *In situ thymidylate synthase activity in CCRF-CEM and CCRF-CEM/P cells*

TS activity was measured immediately after exposure to 150 μM FUra alone for 2 h or 10 μM LV for 4 h and FUra during the last 2 h of LV exposure (time 0) and after 6 additional h in folate-free medium (time 6). Numbers are the percentage of release of tritium compared to untreated control and represent the mean of 3–4 experiments ± SE.

Concentration (μM)		Time (h)	
FUra	LV	0	6
		% of control	% of control
		CCRF-CEM	
150		13.2 ± 1.3	17.3 ± 4.0
150	10	6.8 ± 3.0	11.0 ± 3.6
		CCRF-CEM/P	
150		26.5 ± 1631	25.1 ± 1.7
150	10	33.5 ± 19.0	31.5 ± 7.2

Table 2 *Percentage of thymidylate synthase activity in CCRF-CEM and CCRF-CEM/P cells*

TS activity was measured immediately after exposure to 0.5 μM FdUrd alone for 2 h or 10 μM LV for 4 h, and FdUrd during the last 2 h of LV exposure (time 0), and after 6 additional h in folate-free medium (time 6). Numbers are the percentage of release of tritium compared to untreated controls and represent the mean of 3–4 experiments ± SE.

Concentration (μM)		Time (h)	
FdUrd	LV	0	6
		% of control	% of control
		CCRF-CEM	
0.5		2.1 ± 2.0	8.5 ± 8.4
0.5	10	0.5 ± 0.6	2.2 ± 2.0
		CCRF-CEM/P	
0.5		2.4 ± 2.5	11.2 ± 5.6
0.5	10	3.4 ± 6.5	10.1 ± 13.5

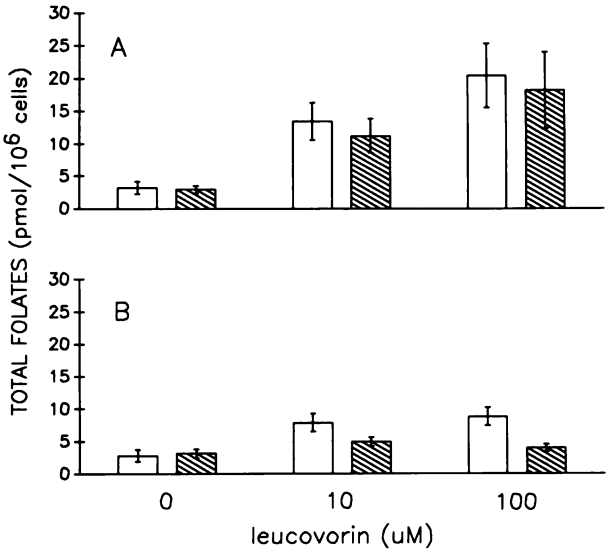

Fig. 3. Effect of 10 and 100 μM LV for 4 h on intracellular total folate pools in CCRF-CEM and CCRF-CEM/P cells. Cells were either analyzed immediately after 4-h exposure (*A*), or after an additional 6-h incubation in folate-free medium (*B*). □, CCRF-CEM cells; ▨, CCRF-CEM/P cells. *Columns*, mean of 3 experiments, each in duplicate; *bars*, SE.

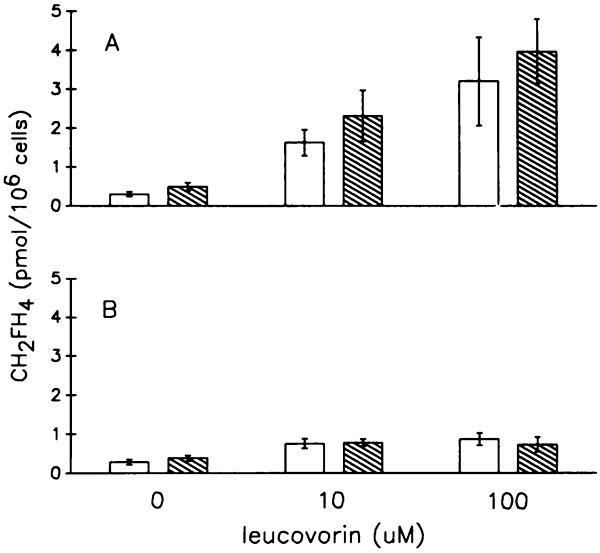

Fig. 4. Effect of 10 and 100 μM LV for 4 h on intracellular CH_2FH_4 pool in CCRF-CEM and CCRF-CEM/P cells. Cells were either analyzed immediately at the end of 4-h exposure (*A*), or after an additional 6-h incubation in folate-free medium. □, CCRF-CEM cells; ▨, CCRF-CEM/P cells. *Columns*, mean of 3 experiments, each in duplicate; *bars*, SE.

Folylpolyglutamates Assay. There was only a slight difference between the two cell lines with regard to the pattern of CH_2FH_4 and FH_4 polyglutamate chain length in untreated cells (Fig. 5). The parent line contained penta- and hexaglutamates with a very small amount of heptaglutamates. The CCRF-CEM/P line also contained penta- and hexaglutamates but not heptaglutamates. After exposure to 10 μM LV for up to 2 h, the time at which the cells would be exposed to fluoropyrimidines, CCRF-CEM cells contained primarily polyglutamates with 5 residues, while the CCRF-CEM/P cells contained primarily folyl-, mono-, and diglutamates.

DISCUSSION

Polyglutamylation is an essential step in folate metabolism. It enables cells to maintain adequate intracellular folate levels,

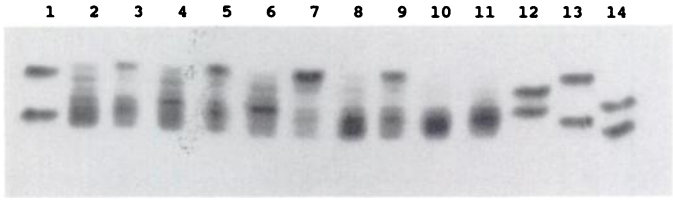

Fig. 5. Polyacrylamide gel electrophoretic separation of folylpolyglutamates complexed with TS and [^{3}H]FdUMP from extracts of CCRF-CEM and CCRF-CEM/P cells. *Lane 1*, Glu_2 and Glu_7 standards; *Lanes 2, 4, 6,* and *8*, CCRF-CEM exposed to 10 μM LV for 30, 60, 120, and 15 min, respectively; *Lanes 3, 5, 7,* and *9*, CCRF-CEM/P exposed to 10 μM LV for 30, 60, 120, and 15 min, respectively; *Lane 10*, CCRF-CEM control; *Lane 11*, CCRF-CEM/P control; *Lane 12*, Glu_3 and Glu_5 standards; *Lane 13*, Glu_1 and Glu_6 standards; *Lane 14*, Glu_4 and Glu_7 standards. Polyglutamate chain length increases from top to bottom of the gel.

and enhances binding of the various folate cofactors to folate-requiring enzymes (3, 4). The availability of a cell line with impaired ability to polyglutamate folates allowed assessment of the importance of CH_2FH_4 polyglutamate formation as a determinant of fluoropyrimidine growth inhibition. At a dose of FUra or FdUrd which produces only a slight decrease in cell growth, the addition of LV further inhibited the cell growth in CCRF-CEM cells, but not in the MTX-resistant CCRF-CEM/P cells. These data are consistent with the hypothesis that the impaired polyglutamylation of CH_2FH_4 may be responsible for the lack of potentiation of the cytotoxic effect of fluoropyrimidines by LV.

The results obtained with measurements of *in situ* TS activity were consistent with the growth inhibition studies in that the addition of 10 μM LV for 4 h to either FUra or FdUrd increased the inhibition of TS activity in CCRF-CEM cells but not in CCRF-CEM/P cells.

LV is a reduced folate which is converted to 5,10-methenyltetrahydrofolate by the enzyme 5,10-methenyltetrahydrofolate synthetase and it then enters the folate pathways (23). Methenyl synthetase activity was found to be identical in both the parental and the MTX-resistant cell line.[6]

The level of CH_2FH_4 was also approximately the same, and increased in a dose-related manner after exposure to LV in both cell lines, confirming that uptake and synthesis of the cofactor is not impaired in CCRF-CEM/P cells.

CH_2FH_4 with longer chain length polyglutamates binds more tightly to TS than do shorter chain polyglutamates, increasing the stability of the ternary complex (6). Thus, for maximal biochemical modulation with LV, it becomes important not only to increase the amount of folate cofactor in the cells, but also to allow the accumulation of longer chain length polyglutamates. With exposure to 10 μM LV, there is clearly a greater distribution of longer chain polyglutamate forms in CCRF-CEM cells than in CCRF-CEM/P cells at all time points up to 2 h (Fig. 5). In CCRF-CEM/P cells, after LV exposure for 2 h, the amount of time before the cells would be exposed to fluoropyrimidines, predominantly short chain CH_2FH_4 polyglutamates were formed (Fig. 5). Under these conditions, FdUMP would be expected to dissociate more readily from the ternary complex (6), and thus limit the enhancement of fluoropyrimidine cytotoxicity. It should be noted that although it appears that the amount of longer chain length polyglutamates decreases in the CCRF-CEM/P line between 60 and 120 min, this is an artifact due to the fact that each lane is loaded with an equal amount of ternary complex, and thus reflects the qualitative distribution of folate polyglutamates in the cells ratner than the actual amounts. Nevertheless, polyglutamylation of CH_2FH_4 and FH_4 is clearly impaired in the CCRF-CEM/P cells exposed to 10 μM LV for 2 h compared to CCRF-CEM cells. The former contains mainly Glu_2 forms, while the latter contains predominantly Glu_5, under the same experimental conditions. Given this difference in distribution of polyglutamate forms, one would also expect better retention of folates in the parent CCRF-CEM cells than in the MTX-resistant CCRF-CEM/P cells, as was shown by the higher folate levels measured in the parent cell line after 6 h in folate-free media (Fig. 3*B*). The rapid decrease in retained folates in the polyglutamylation-deficient CCRF-CEM/P cells could also be a factor in reducing the efficacy of leucovorin modulation of fluoropyrimidine cytotoxicity in this cell line.

These results have important implications for the clinical use of LV with FUra or FdUrd. Cells unable to increase levels of long chain length CH_2FH_4 polyglutamates ($N = 3$–7) because of impaired ability to polyglutamate reduced folates, will not express enhanced sensitivity to this combination because the FdUMP-TS-CH_2FH_4 ternary complex formed will dissociate more readily in the absence of these polyglutamates. Both small cell lung cancer cell lines (25) as well as acute myelocytic leukemia cells (26) and sarcoma cell lines (27) are unable to form high levels of MTX-polyglutamates *in vitro*, and may also be unable to form appreciable levels of folate polyglutamates as well. It is of interest that these human neoplasms are considered to be refractory to fluoropyrimidine therapy (28, 29). We plan to extend these studies to specimens of colorectal carcinoma, to determine if sensitivity or resistance to LV with fluoropyrimidines is correlated with the inability of the patient's tumor cells to increase levels of CH_2FH_4 polyglutamates after exposure to LV.

[6] M. X. Li and J. R. Bertino, manuscript in preparation.

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