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COMPARTMENTATION OF INTRACELLULAR FOLATES

FAILURE TO INTERCONVERT TETRAHYDROFOLATE COFACTORS TO DIHYDROFOLATE IN MITOCHONDRIA OF L1210 LEUKEMIA CELLS TREATED WITH TRIMETREXATE

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Abstract—Following exposure of L1210 leukemia cells to antifolates, tetrahydrofolate-dependent purine and pyrimidine biosyntheses are blocked despite the presence of the major portion of tetrahydrofolate cofactors. Previous studies from this laboratory demonstrated that this cannot be due to direct inhibition of thymidylate synthase by dihydrofolate polyglutamates or other endogenous folates and suggested that this phenomenon is due to compartmentation of tetrahydrofolate cofactors unavailable for interconversion and/or oxidation when dihydrofolate reductase activity is abolished by antifolates. The present paper evaluates the possibility that tetrahydrofolate cofactors in subcellular organelles, in particular, mitochondria, are unavailable for oxidation by thymidylate synthase. Particulate and cytosolic fractions were obtained from L1210 cells following homogenization and differential centrifugation. The crude mitochondrial fraction contained 20.1% of the total folate pool and included 5-formyltetrahydrofolate, 10-formyltetrahydrofolate and tetrahydrofolate in proportions similar to intact cells. The cytosolic fraction had an increased proportion of tetrahydrofolate and decreased proportions of 5-formyl- and 10-formyltetrahydrofolate relative to intact cells or the particulate fraction. Exposure of cells to 10 μM trimetrexate for 30 min produced ~45% interconversion of tetrahydrofolate cofactors to dihydrofolate in the cytosolic fraction, a level much greater than that observed in whole cell extracts (25–30%), but had no effect on folate pools in the crude mitochondrial fraction. These data indicate that subcellular compartmentation accounts, in part, for the failure to oxidize tetrahydrofolate cofactors to dihydrofolate in the presence of antifolate levels that abolish dihydrofolate reductase activity.

The major portion of intracellular tetrahydrofolate cofactors is preserved following exposure to antifolate levels that totally suppress folate-dependent biosynthetic processes [1–6]. Studies by Seither *et al.* [6] suggest that the failure to totally deplete tetrahydrofolate cofactors after antifolates is due to compartmentation of intracellular folates such that some fraction(s) is not available for oxidation by thymidylate synthase. Several possible types of compartmentation have been suggested: (i) cell cycle related compartmentation in which there are non-S phase cells that lack sufficient thymidylate synthase catalytic activity to oxidize tetrahydrolate cofactors [6–9], (ii) compartmentation in which component folates are bound to proteins that could restrict [6, 10–12] or facilitate [13, 14] their utilization, and (iii) compartmentation due to sequestration of folates in subcellular organelles in which interconversion of tetrahydrofolate cofactors to dihydrofolate does not occur [15, 16].

The subcellular compartmentation of intracellular folates has been described for rat liver [17, 18], mouse brain [19, 20] and liver [21], and guinea pig liver and small intestine [22, 23]. Intracellular folates have been localized primarily to the cytosol (50–80%) and mitochondria (20–50%) in these tissues. Furthermore, Horne *et al.* [16] showed that exposure to nitrous oxide produces marked changes in the composition of cytosolic tetrahydrofolate cofactors without any effect on mitochondrial folates. This raised the possibility that mitochondrial folates may represent a compartment of tetrahydrofolate cofactors in which oxidation to dihydrofolate does not occur in the presence of antifolates.

The present study describes the effects of trimetrexate, a lipophilic dihydrofolate reductase inhibitor [24], on folates from cytosolic and mitochondrial fractions obtained from L1210 leukemia cells. The data indicate that tetrahydrofolate cofactors in mitochondria do not interconvert to dihydrofolate after suppression of dihydrofolate reductase by trimetrexate. These observations provide one explanation for the incomplete depletion of tetrahydrofolate cofactor pools within these cells after exposure to antifolates.

METHODS

Chemicals. Cytochrome *c*, EDTA, β-mercaptoethanol (BME†), Tris–HCl, dihydrofolate, tetrahydrofolate, 5-formyltetrahydrofolate and 5-methyltetrahydrofolate were purchased from the Sigma Chemical Co. 5,10-Methenyltetrahydrofolate was prepared from 5-formyltetrahydrofolate by acidification to pH 2 with HCl in 0.1 M KPO_4 buffer

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† Abbreviations: BME, β-mercaptoethanol; and PABA-glu, *p*-aminobenzoylglutamic acid.

containing 1% BME [25]. 10-Formyltetrahydrofolate was prepared by neutralizing 5,10-methenyltetrahydrofolate to pH 7.5 [25]. [^{3}H]Folic acid (20 Ci/mmol) and [^{3}H]leucovorin (20 Ci/mmol) were obtained from Moravek Biochemicals and purified by HPLC as described previously [1]. Trimetrexate glucuronate was provided by Warner-Lambert Pharmaceuticals.

Cell culture. L1210 murine leukemia cells were grown in RPMI 1640 medium supplemented with 10% bovine serum (Hyclone Laboratories), 10 mM L-glutamine, 20 μM BME, penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were passaged every 2–3 days to maintain logarithmic growth and harvested at ~1 × 10^6 cells/mL.

Cell fractionation. L1210 leukemia cells were washed three times in ice-cold 0.9% NaCl and the pellet was resuspended in approximately 10 vol. of hypotonic lysis medium consisting of 10 mM Tris–HCl (pH 7.8), 5 mM $MgCl_2$ and 2 mM $CaCl_2$ at 0°. After 30 min, ascorbate and BME were each added to a final concentration of 1% and cells were disrupted in a tight-fitting Dounce homogenizer (Wheaton, pestle A) with 50 strokes. Sucrose was added to a final concentration of 0.25 M and the homogenate layered over 5 mL of 25% sucrose and centrifuged at 600 *g* for 10 min to remove cell debris, intact cells and nuclei as confirmed by light microscopy. The supernatant was collected by aspiration and centrifuged at 10,000 *g* for 10 min at 0° to prepare crude mitochondrial and cytosolic fractions.

Succinate–cytochrome *c* reductase was assayed in cell homogenates prepared with 0.25 M sucrose, as described above, but without ascorbate or BME. Pellet fractions were resuspended in lysis buffer with 0.25 M sucrose in tubes immersed in ice and subjected to six 10-sec bursts of sonic oscillation (Fisher model 300) at 35% of maximum output (micro tip). Total protein was determined by the Bradford protein assay from Bio-Rad Laboratories using bovine serum albumin as a standard [26]. Succinate–cytochrome *c* reductase activity was measured according to the method of King [27] and used as a marker for mitochondria. The succinate–cytochrome *c* reductase reaction mixture consisted of 700 μL of 0.1 M KPO_4 buffer (pH 7.4) containing 1 mM KCN, 100 μL of 0.5 mM cytochrome *c*, 100 μL of 200 μM succinate and 100 μL of sample at 25°. The change in absorbance at 550 nm was linear for 3–5 min and was proportional to the protein concentration for both pellet and supernatant fractions.

Analysis of intracellular folates. Intracellular folate pools were radiolabeled by growing L1210 leukemia cells for 72 hr in folate-free medium containing dialyzed serum and [^{3}H]folic acid (2.3 μM, 500–1000 dpm/pmol). [^{3}H]Folates were extracted from the various cellular fractions by boiling for 2 min in nitrogen-saturated maleate buffer (0.1 M, pH 6) containing 2% ascorbate and 2% BME as described previously [1, 6]. The extracts were incubated for 4 hr at 37° with hog kidney conjugase to cleave folypolyglutamates to their respective monoglutamates [28]. The resultant extract was analyzed by reverse-phase HPLC on a C-18 column eluted with sodium acetate (0.1 M, pH 5.5) and 2.55% acetonitrile for 9 min followed by a linear gradient to 6.75% acetonitrile over 10 min at a flow rate of 2 mL/min [1]. Radioactivity in 0.5-min fractions was detected by liquid scintillation spectroscopy.

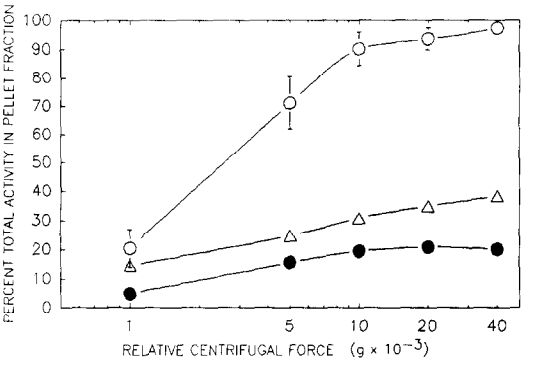

Fig. 1. Differential centrifugation of L1210 leukemia cell homogenates. Folate pools in L1210 cells in logarithmic growth were radiolabeled with [^{3}H]folic acid in the absence of nonlabeled folic acid in the medium or serum for 72 hr. Cells were then homogenized as described in Methods and centrifuged as indicated for 10 min. The data are plotted as the percent of total activity which was pelleted at each speed for protein (△), succinate–cytochrome *c* activity (○) and [^{3}H]folates (●). Data are means ± SEM for at least three separate experiments performed on different days.

The intracellular folate pools are uniformly labeled under these conditions with an intracellular concentration of 6.2 pmol/10^6 cells. A total of 1–2 × 10^8 cells was extracted per experiment to obtain 30–100 dpm of [^{3}H]folates in each fraction for analysis by HPLC. Seven folate moieties were identified by co-elution with synthetic standards as described previously [1]. Recovery of 10-formyltetrahydrofolate is variable under these conditions due to interconversion to 5-formyltetrahydrofolate and tetrahydrofolate. Low levels of 5-methyltetrahydrofolate are detected in L1210 cells and recovery of this cofactor is greater than 90% [6]. This cofactor does not accumulate in L1210 leukemia cells grown in RPMI 1640 medium presumably due to the high methionine concentration [29].

RESULTS

Subcellular fractionation. Hypotonic extraction followed by 10–50 strokes in a Dounce homogenizer achieved complete disruption of cells. Inadequate cellular disruption was seen even with up to 100 homogenizing strokes when hypotonic medium was not employed. Differential centrifugation was then used to isolate crude cytosolic and mitochondrial fractions from cell homogenate. Figure 1 shows the fraction of [^{3}H]folates, protein and succinate–cytochrome *c* reductase activity which sedimented in 10 min from crude L1210 cell homogenate with increasing centrifugation speeds. Virtually all the succinate–cytochrome *c* reductase activity was sedimented at 10,000–20,000 *g* for 10 min as expected

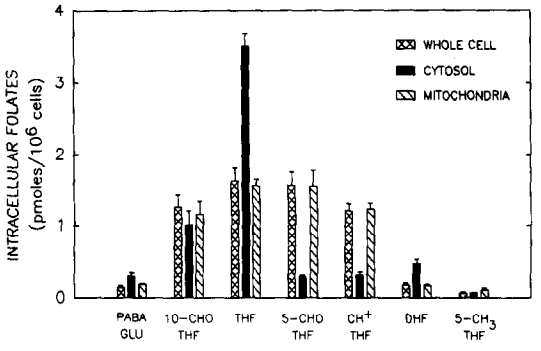

Fig. 2. HPLC analysis of [^{3}H]folates obtained from whole cells following exposure to hypotonic medium, crude cytosolic fractions and mitochondrial fractions. The data are presented as picomoles of [^{3}H]folates per 10^6 cells. The seven folyl derivatives shown account for ≥90% of the total radioactivity recovered. Data are means ± SEM for six experiments. Abbreviations are: PABA-glu, *p*-aminobenzoylglutamic acid; 5-CHO-THF, 5-formyltetrahydrofolate; 10-CHO-THF, 10-formyltetrahydrofolate; THF, tetrahydrofolate; CH^+-THF, 5,10-methenyltetrahydrofolate; DHF, dihydrofolate; and 5-CH_3-THF, 5-methyltetrahydrofolate.

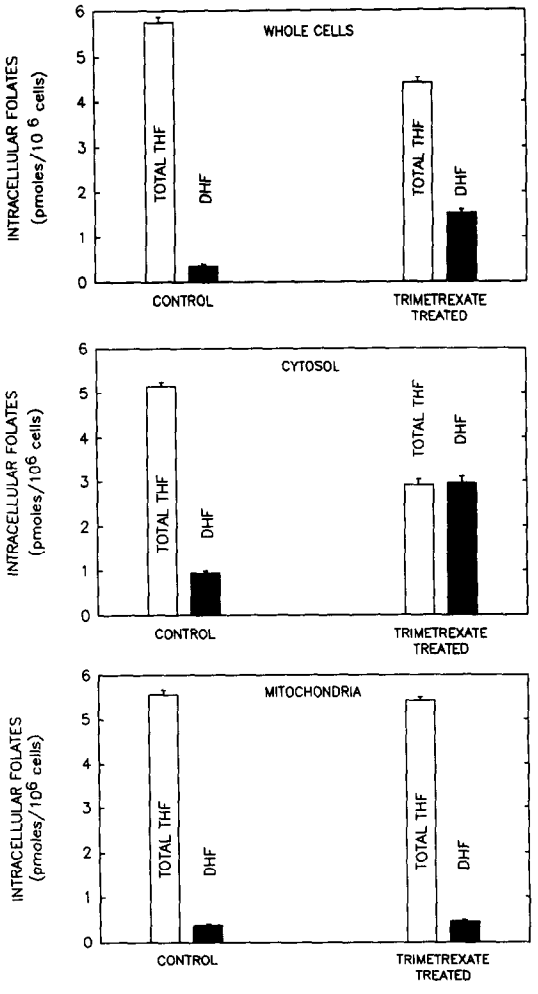

Fig. 3. Effects of a 30-min exposure of L1210 leukemia cells to 10 μM trimetrexate on tetrahydrofolate cofactor interconversion to dihydrofolate in whole cells (upper panel), crude cytosol (middle panel), and crude mitochondrial fractions (lower panel). Total tetrahydrofolates (THF) represents the sum of the tetrahydrofolate cofactors measured as compared to dihydrofolate (DHF) with all folates expressed as picomoles per 10^6 cells. Data are means ± SEM from at least six separate experiments performed on different days.

for mitochondria. Maximal co-sedimentation of [^{3}H] folates with this mitochondrial enzymatic activity consisted of 20.1% of the total cellular radiolabeled folates. The total intracellular concentration of folates is ~6.2 pmol/10^6 cells under these conditions.

Non-specific binding of intracellular [^{3}H]folates to the crude mitochondrial fraction was not detected. The crude mitochondrial pellet did not bind either [^{3}H]folic acid or [^{3}H]5-formyltetrahydrofolate when added to the lysis medium prior to extraction. Further, tritiated endogenous folylpolyglutamates extracted from L1210 cells were added to the lysis medium during processing of unlabeled cells, and less than 1% of the endogenous folates were pelleted in the crude mitochondrial fraction.

HPLC analysis of radiolabeled folates. The profiles of individual folates for whole cells (after exposure to hypotonic medium) or crude cytosolic and mitochondrial fractions are shown in Fig. 2. The standard extraction protocol for whole cells gives a typical pattern of 10-formyltetrahydrofolate (~20%), tetrahydrofolate (~25%), 5-formyltetrahydrofolate (~20%) and 5,10-methenyltetrahydrofolate (~20%) with minor quantities (1–3%) of dihydrofolate, 5-methyltetrahydrofolate and *p*-aminobenzoylglutamic acid (PABA-glu) [1, 6]. This pattern was unchanged by preincubating cells in lysis buffer. The profile of the crude mitochondrial fraction was similar to that of whole cells. Conversely, cytosolic extracts had a different pattern with increased tetrahydrofolate (~53%) and decreased 5-formyltetrahydrofolate (~5%) and decreased 5,10-methenyltetrahydrofolate (~7%). There was also a small but significant increase in the amount of dihydrofolate and PABA-glu in cytosolic extracts even in the presence of ascorbate and BME in the extraction medium. This relative shift in the folate profile suggests interconversion of 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate to tetrahydrofolate under the conditions used to prepare the cytosolic fraction.

Effect of trimetrexate on intracellular folates. Previous studies have shown that trimetrexate produces partial depletion of cellular tetrahydrofolate factors reaching a minimum level within a few minutes in L1210 leukemia cells [1, 6]. The effects of a 30-min exposure to trimetrexate (10 μM) on folates in whole cells and in crude cytosolic and mitochondrial fractions are shown in Fig. 3. Cells extracted under standard conditions or after exposure to hypotonic medium (Fig. 3, upper panel) showed the typical 25–30% interconversion of tetrahydrofolate cofactors to dihydrofolate noted previously [1, 6]. Interconversion of tetrahydrofolate

cofactors to dihydrofolate was higher in the crude cytosolic fraction where ~45% of the total folate pool was oxidized to dihydrofolate (Fig. 3, middle panel). There was negligible interconversion of tetrahydrofolate cofactors to dihydrofolate in the crude mitochondrial fraction (Fig. 3, lower panel). Finally, there was no difference in interconversion in the crude mitochondrial fraction even when cells were exposed to 10-fold higher levels of trimetrexate (100 μM).

DISCUSSION

This study describes the subcellular compartmentation of radiolabeled folates in L1210 leukemia cells and the effects of a dihydrofolate reductase inhibitor, trimetrexate, on these pools. Previous studies in a variety of tissues have localized folates primarily in cytosol and mitochondria [15–23]. The procedure used here produced a crude fraction of L1210 leukemic cells enriched for mitochondrial enzyme activity (succinate–cytochrome *c* reductase) that contained 20% of the extractable intracellular folates. It is unlikely that this represents non-specific binding of [^{3}H]folyl derivatives to mitochondria during preparation since neither purified folate monoglutamates (5-formyltetrahydrofolate and folic acid) nor endogenous radiolabeled folylpolyglutamyl derivatives bound to crude mitochondrial fractions during the extraction procedure.

Matherly *et al.* [14] reported recently that mitochondrial fractions obtained from L1210 cells contain only ~3.5% of the total intracellular folates. In these studies, cells were folate depleted by growth in folate-free medium supplemented by adenosine and thymidine and then radiolabeled with low concentrations of [^{3}H]5-formyltetrahydrofolate rather than [^{3}H]folic acid. Furthermore, the mitochondrial fraction was prepared by a different procedure. Thus, it is difficult to compare these results quantitatively due to the differences in folate source, initial folate levels, as well as extraction techniques.

The present study revealed clear differences in the relative distribution of folate cofactors from the cytosolic and mitochondrial fractions. Cytosol had relatively higher concentrations of tetrahydrofolate and lower concentrations of 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate than were found in the mitochondrial or whole cell extracts. Dihydrofolate levels were also higher in the cytosolic fraction even in the presence of BME and ascorbate. In contrast, mitochondrial extracts from L1210 cells had higher concentrations of 5-formyl-, 10-formyl-, 5,10-methenyltetrahydrofolate and tetrahydrofolate with negligible dihydrofolate and were similar in composition to the standard whole cell extracts. The data suggest that [^{3}H]folate cofactors in the crude mitochondrial fraction are protected from degradation during the fractionation, whereas cytosolic tetrahydrofolate cofactors undergo significant interconversion and oxidation. The relatively high proportion of 5-formyltetrahydrofolate observed in these cell extracts may result from interconversion of 10-formyltetrahydrofolate and may represent a *de novo* folate pool. Stover and Schirch [30, 31] suggested recently that 5-formyltetrahydrofolate may be an important physiologic folate involved in the regulation of one-carbon metabolism within cells. Studies with rat hepatocytes previously showed differences in the pattern of folates in cytosol compared to mitochondria [16, 18]. Those studies found, however, that mitochondria had increased levels of 10-formyltetrahydrofolate relative to the cytosolic compartment. It is unclear whether this is due to actual differences in subcellular folate distribution between these cell types or differences in extraction methods.

The current study demonstrates clearly that the [^{3}H]folates in crude cytosolic and mitochondrial fractions also differ in response to trimetrexate. Cytosolic tetrahydrofolate cofactors underwent rapid oxidation to dihydrofolate while there was no detectable oxidation of tetrahydrofolate cofactors to dihydrofolate in the crude mitochondrial fraction. The lack of availability of mitochondrial folates for interconversion after metabolic perturbation is consistent with the results of Horne *et al.* [16] who found that mitochondrial folate pools are unaffected by nitrous oxide exposure of up to 18 hr whereas cytosolic folates undergo marked interconversions.

The present data substantiate the previous suggestion from this laboratory that intracellular folates are compartmentalized such that some tetrahydrofolate cofactor pools are not susceptible to oxidation to dihydrofolate following exposure to antifolates [6]. This explains, in part, the failure to deplete tetrahydrofolate cofactors after an antifolate treatment that totally abolishes folate-dependent purine and pyrimidine biosyntheses. The cytosolic tetrahydrofolate cofactors are labile and readily oxidized to dihydrofolate, whereas folates in the crude mitochondrial fraction are unaffected. It is unlikely that the failure to interconvert tetrahydrofolate cofactors to dihydrofolate in mitochondria is due to poor penetration of trimetrexate into the organelle because of the high lipid solubility of this antifolate and the unchanged results when the trimetrexate concentration was increased to 100 μM. However, interconversion was incomplete even within the "sensitive" cytosolic compartment where only 45–50% of the total folate pool was interconverted to dihydrofolate. This phenomenon is consistent with our recent observation that there are non-S phase cells that do not interconvert tetrahydrofolate cofactors to dihydrofolate because of a very low level of thymidylate synthase catalytic activity [9]. The combined effect of subcellular compartmentation and cell cycle phenomena may account for the failure to interconvert tetrahydrofolate cofactors to dihydrofolate in the presence of antifolates that abolish dihydrofolate reductase activity.

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