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GROWTH INHIBITION BY HOMOFOLATE IN TUMOR CELLS UTILIZING A HIGH-AFFINITY FOLATE BINDING PROTEIN AS A MEANS FOR FOLATE INTERNALIZATION

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Abstract—A subline (JT-1) of L1210 mouse leukemia cells that contains elevated levels of a high-affinity folate binding protein is sensitive to growth inhibition by homofolate. Inhibition was observed at nanomolar concentrations of folate or 5-formyltetrahydrofolate where the high-affinity binding protein is the predominant uptake route for folate compounds. At 1.0 nM folate, inhibition of growth by 50% occurred at 0.7 nM homofolate, and maximal inhibition exceeded 90% at homofolate concentrations above 10 nM. Homofolate also inhibited the uptake of 1.0 nM [^{3}H]folate by L1210/JT-1 cells in 72-hr cultures, and the extent of uptake inhibition by 1.0 and 20 nM homofolate was comparable to the inhibition of cell growth by the same concentrations of homofolate. At a growth-limiting concentration of 5-formyltetrahydrofolate (0.5 nM), half-maximal inhibition of L1210/JT-1 cell growth occurred at 1.0 nM homofolate. When excess concentrations of folate (5 μM) or 5-formyltetrahydrofolate (0.5 μM) were added to the medium, no growth inhibition was observed for homofolate at concentrations up to 100 μM. Parental cells lacking the folate binding protein did not respond to homofolate either at growth-limiting (0.5 μM) or excess (5.0 μM) levels of folate. Binding measurements showed that homofolate has a high affinity for the folate-binding protein (K_i = 0.03 nM) but interacts poorly with the reduced-folate transport system (K_i = 203 μM). These results indicate that homofolate inhibits the growth of L1210 cells when intracellular folates are acquired via the high-affinity folate binding protein. The basis for this inhibition appears to be competition by homofolate for substrate binding and internalization.

Tumor cells become resistant to methotrexate by a variety of mechanisms including decreased cellular uptake via carrier-mediated transport systems [1–3]. The usual function of these transport systems is to mediate the uptake of folate compounds, but flexibility in binding specificity is generally observed which allows most systems to also transport folate analogs such as methotrexate. As a consequence, resistance to methotrexate that occurs via decreased transport has a high likelihood of also reducing the uptake of folate compounds needed for cell growth. Survival of tumor cells, therefore, hinges on compromising the transport of methotrexate without eliminating the ability of cells to acquire sufficient folate cofactors for growth. A mechanism by which tumor cells might achieve these transport changes is to selectively down-regulate a system which transports methotrexate with a high efficiency and to utilize a second transport system which shows a lower transport efficiency for methotrexate.

Sublines of mouse L1210 cells have been isolated which grow at low concentrations of folate or 5-formyltetrahydrofolate and have acquired elevated levels of a high-affinity folate binding protein [4, 5]. Similar high-affinity binding proteins have been observed previously in KB [6, 7], MA104 [8], and various other [6] cells and have been implicated in the cellular internalization of folate compounds. Transport via this binding protein was proposed as the basis for growth of L1210 cells under low folate conditions [4]. The binding protein in L1210 cells exhibits a high affinity for folate (K_d = 0.07 nM), 5-methyltetrahydrofolate (K_i = 13 nM) and 5-formyltetrahydrofolate (K_i = 45 nM) relative to methotrexate (K_i = 325 nM) [4]. This preference for folate and reduced folate compounds relative to methotrexate is much different from the specificity of the reduced-folate carrier system of L1210 cells [2, 9, 10], which transports methotrexate and reduced-folate compounds with about equal facility (K_t = 1–5 μM) but is much less able to transport folate (K_t = 100–200 μM). These differences in specificity suggest that resistance to methotrexate should develop in sublines of L1210 cells which down-regulate the reduced-folate carrier system and acquire folate compounds by a compensating up-regulation of the high-affinity folate-binding protein system.

Antifolate compounds which can utilize or interfere with the activity of high-affinity folate binding proteins may be useful in killing tumor cells which rely on these binding proteins for folate acquisition. One possible folate analog with these characteristics is homofolate, which contains an additional methylene group between the C-9 and N-10 positions of folate [11]. Reduction of homofolate to tetrahydrohomofolate occurs via dihydrofolate reductase in mouse leukemia cells [12], and this leads to folate coenzyme analogs which inhibit purine [13] and pyrimidine [14] biosynthesis. The efficacy, however, of homofolate [12, 13, 15, 16], dihydrohomofolate [12, 16], tetrahydrohomofolate [12, 13, 16, 17], and

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5-methyltetrahydrohomofolate [17] in killing either sensitive or methotrexate-resistant tumor cells is low compared to other antifolate compounds, presumably due to inefficient conversion to polyglutamate forms [18, 19]. In the present study, homofolate was examined as a possible means for inhibiting the growth of a subline of L1210 cells (L1210/JT-1) [4] under conditions where folate acquisition occurs via the high-affinity folate binding protein. Homofolate was found to be a potent growth inhibitor under these conditions and the mechanism of inhibition appears to involve competition for folate internalization via the high-affinity binding protein.

MATERIALS AND METHODS

Chemicals. [3′,5′,7,9-^{3}H]Folic acid (40 Ci/mmol) and [3′,5′,7-^{3}H]methotrexate (20 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA) and stored at −80°. Folic acid, [6*R*,6*S*]-5-formyltetrahydrofolate (folinate), methotrexate, and HEPES* were obtained from the Sigma Chemical Co. (St. Louis, MO). Homofolate was a gift of Dr. John H. Mangum, Brigham Young University.

Purification of labeled substrates. [^{3}H]Folate (24,000,000 cpm/nmol) was purified on Baker-flex cellulose sheets at 23° in 50 mM sodium HEPES, pH 7.5, as described previously [20] and stored in HEPES-buffered saline (HBS) containing 2% ethanol for up to 2 weeks at −20°. [^{3}H]Methotrexate (100,000 cpm/nmol) was purified by the same procedure and stored for up to 6 weeks at −20°.

Growth of cells. Parental L1210 mouse cells were grown in RPMI 1640 medium supplemented with 2.5% fetal bovine serum and 100 units each of penicillin and streptomycin. Folate-sufficient L1210 cells were grown in folate-free RPMI 1640 medium supplemented with 2.5% folate-depleted serum [4], antibiotics, and 500 nM folate. L1210/JT-1 cells were grown in folate-free RPMI 1640 medium supplemented with folate-depleted serum, antibiotics, and 1 nM folate. Folate-depleted L1210 and L1210/JT-1 cells were derived from folate-sufficient cells after growth for 3–4 generations (one transfer) in folate-free medium. Large cultures (500 mL) of L1210/JT-1 cells were grown in sealed 1-liter flasks with gentle shaking at 37°. Cells were harvested by centrifugation at 1000 *g* (5 min, 4°), washed with HBS, 20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, pH 7.4, with NaOH, and suspended to a density of 3×10^7/mL.

The growth response of cells to various concentrations of homofolate or methotrexate was measured in 96-well plates by the procedure of Mosmann [21]. Each well contained 1000 cells and the desired additions in a final volume of 150 μL. Plates were developed with a tetrazolium dye assay after 4–5 days and were quantified in a plate reader at 570 nm [21]. Data points in growth studies represent the mean of three separate experiments performed in duplicate. Standard deviations were less than 20%.

* Abbreviations: HEPES, *N*-hydroxyethylpiperazine-*N*′-ethanesulfonate; and HBS, HEPES-buffered saline.

Transport and binding measurements. Methotrexate transport was measured in duplicate assay mixtures containing L1210/JT-1 cells (3×10^7), the desired additions, [^{3}H]methotrexate (100,000 cpm/nmol), and HBS in a final volume of 1.0 mL. After incubation for 4 min at 37°, the cells were chilled to 0°, diluted with 7 mL of ice-cold saline (160 mM NaCl, 1 mM sodium phosphate, pH 7.4), recovered by centrifugation at 1000 *g* (5 min, 4°), resuspended in 0.5 mL saline, and analyzed for radioactivity. Uptake at 0° served as the control.

Binding of folate was measured in duplicate assay mixtures containing L1201/JT-1 cells (3×10^7/mL), 5 nM [^{3}H]folate (24,000,000 cpm/nmol), the desired additions, and HBS in a final volume of 1.0 mL. After incubation for 10 min at 37°, the cells were chilled to 0°, diluted with 7 mL of ice-cold saline, recovered by centrifugation, and analyzed for radioactivity as described above. Uptake in samples containing 1 μM unlabeled folate served as the control. Protein concentrations were measured by the biuret reaction [22] using bovine serum albumin as the standard. Kinetic constants for transport or binding were the mean of three or more separate experiments whose standard deviations varied by less than 20%.

RESULTS

Effect of homofolate on cell growth. Parental L1210 cells require a relatively high extracellular concentration of folate (above 250 nM) to achieve optimal cell growth, and the basis for this requirement has been attributed to the relatively low capacity of these cells for transporting folic acid [2, 9, 10]. L1210/JT-1 cells, in contrast, have the capacity to grow at nanomolar concentrations of folate [4]. The latter cells contain elevated levels of a high-affinity folate binding protein which is more efficient than the existing reduced-folate transport system in retrieving folate compounds at low extracellular concentrations. Uptake at folate concentrations between 0.5 and 50 nM could be attributed to the high-affinity binding protein, whereas folate acquisition in the 100–2000 nM range occurred predominantly via the reduced-folate carrier system. The acquisition of a high-affinity binding protein system enabled L1210/JT-1 cells to grow at low concentrations of folate, and it also suggested that antifolate compounds which are analogs of folate might be able to inhibit the growth of these cells. One compound selected as a potential inhibitor of high-affinity binding protein function was homofolate.

Homofolate was examined for an effect on the growth of L1210/JT-1 cells over a range of extracellular concentrations of folate or 5-formyltetrahydrofolate. In medium containing folate at a concentration (1.0 nM) where uptake proceeds via the high-affinity folate binding protein (Fig. 1A), L1210/JT-1 cell growth was inhibited 50% at 0.7 nM homofolate, and inhibition exceeded 90% at higher concentrations of homofolate. In contrast, L1210/JT-1 cells grown at a high folate concentration (5 μM) where uptake proceeds primarily via the reduced-folate carrier system exhibited no growth inhibition by homofolate at concentrations up to 100 μM (Fib. 1B). Homofolate was not an effective inhibitor of

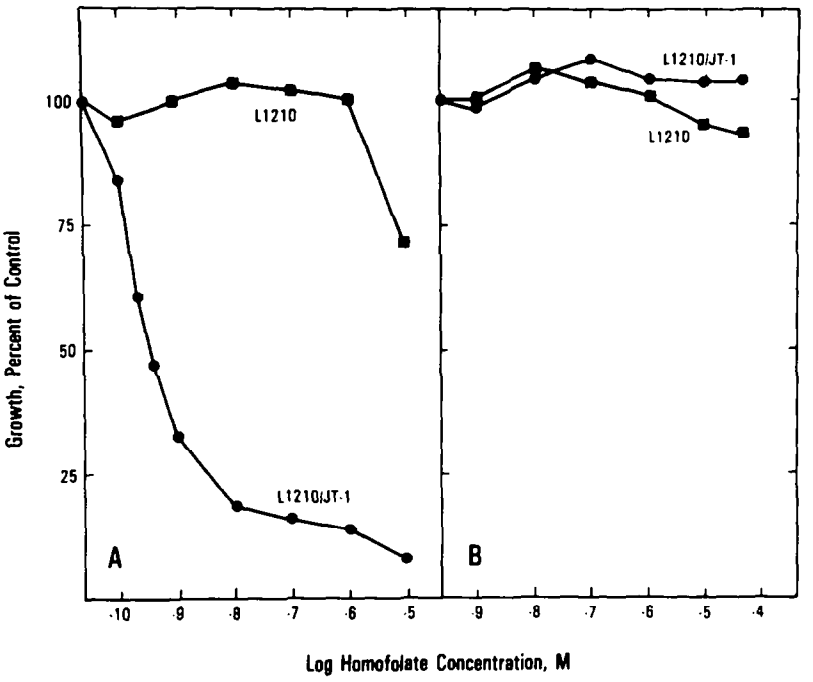

Fig. 1. Growth response of L1210 and L1210/JT-1 cells to various concentrations of homofolate at limiting (A) and excess (B) levels of folate. Folate-depleted L1210 and L1210/JT-1 cells were grown in 96-well plates in folate-free RPMI 1640 medium containing 2.5% folate-depleted fetal bovine serum, antibiotics, folate as indicated, and various concentrations of homofolate. Growth was measured after 4–5 days by a tetrazolium dye assay as described in Materials and Methods. Reduced tetrazolium formation (A_{570} nm) of samples without homofolate was 1.0 ± 0.2, and all values were corrected by a control (A_{570} = 0.10 ± 0.05) without folate. (A) Limiting concentration of folate: L1210 cells, 500 nM; L1210/JT-1 cells, 1 nM. (B) Excess concentration of folate, 5 μM.

parental cell growth, either at a limiting (0.5 μM) (Fig. 1A) or an excess (5 μM) concentration of folate (Fig. 1B).

The growth of L1210/JT-1 cells was also inhibited by homofolate in medium containing a low concentration (0.5 nM) of 5-formyltetrahydrofolate (Fig. 2A). Inhibition was half-maximal at a homofolate concentration of 1.0 nM, but in this case maximal inhibition (70%) was less than with cells grown with limiting folate. Since the reduced-folate carrier system has a much higher affinity for 5-formyltetrahydrofolate than folate [2, 9, 10], a portion of the folate requirement of the L1210/JT-1 cells, even at 0.5 nM 5-formyltetrahydrofolate, was apparently being fulfilled by uptake via the reduced-folate carrier system. No growth inhibition by homofolate was observed in either cell line at an excess concentration (5 μM) of 5-formyltetrahydrofolate (Fig. 2B).

Interaction of homofolate with the high-affinity folate binding protein. The inhibition of L1210/JT-1 cell growth by homofolate suggested that homofolate was either entering cells and being metabolized to cytotoxic analogs of folate coenzymes or was decreasing the intracellular availability of folate compounds by inhibiting uptake. In either case, since homofolate was effective only in cells grown at low concentrations of folate or 5-formyltetrahydrofolate, the effects of homofolate could be attributed to an interaction with the high-affinity folate binding protein. Binding measurements showed that homofolate has a high affinity for the folate binding protein. Dixon plots of binding of 5.0 nM [^{3}H]folate in the presence of increasing concentrations of homofolate were linear and showed that homofolate was more effective than folate in competing for the binding site on this protein. Employing the Dixon equation and a K_d for folate of 0.07 nM [4], the calculated K_i for the binding of homofolate was 0.03 nM (Table 1). For comparison, K_i values for the binding of folate, methotrexate, and 5-formyltetrahydrofolate are also listed in Table 1.

Interaction of homofolate with the reduced-folate transport system. Homofolate is a weak inhibitor of transport via the reduced-folate carrier system. When influx of 5.0 μM [^{3}H]methotrexate was measured in the presence of increasing concentrations of homofolate, Dixon plots of the data were linear and gave a K_i for homofolate of 203 μM (Table 1). Folate analyzed in the same fashion inhibited [^{3}H]methotrexate influx with a K_i value of 219 μM.

Effect of homofolate on the uptake of folate by L1210/JT-1 cells in culture. The tight binding of homofolate to the high-affinity folate binding protein of L1210/JT-1 cells suggested that homofolate was inhibiting cell growth by decreasing the ability of the cells to take up folate or 5-formyltetrahydrofolate. This possibility was supported further by direct uptake measurements with L1210/JT-1 cells in culture (Table 2). Uptake of 1.0 nM [^{3}H]folate by cells grown for 72 hr at 37° was reduced 52% by a con-

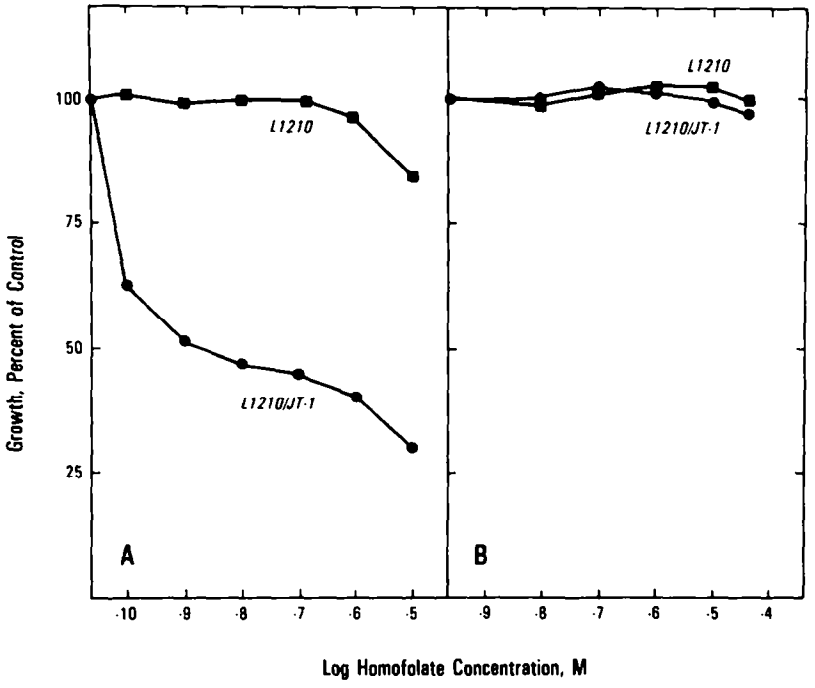

Fig. 2. Growth response of L1210 and L1210/JT-1 cells to various concentrations of homofolate at limiting (A) and excess (B) levels of 5-formyltetrahydrofolate. Cells were grown and analyzed as described in the legend to Fig. 1 except that 5-formyltetrahydrofolate was employed in place of folate. (A) Limiting concentration of 5-formyltetrahydrofolate: L1210 cells, 5 nM; L1210/JT-1 cells, 0.5 nM. (B) Excess concentration of 5-formyltetrahydrofolate, 5 μM.

Table 1. Comparative affinity of folate compounds for the high-affinity folate binding protein and the reduced-folate transport system of L1210/JT-1 cells

	K_i (nM)	
Folate compound	High-affinity binding protein	Reduced-folate transport system
Methotrexate	325	4,500
5-Formyltetrahydrofolate	45	3,200
Folate	0.09	219,000
Homofolate	0.03	203,000

The inhibition constant (K_i) values for the binding protein were measured in HBS, pH 7.4, in cells that had been incubated with 5.0 nM [^{3}H]folate for 10 min at 37° in the presence of various concentrations of the indicated folate compound. Calculations of K_i were derived from Dixon plots of the data and a K_d for folate of 0.07 nM. Inhibition of methotrexate transport via the reduced-folate transport system was measured in cells that had been incubated with 5.0 μM [^{3}H]methotrexate and various concentrations of folate compound for 4 min at 37° in HBS, pH 7.4. K_i values were calculated from Dixon plots of the data using a K_i for methotrexate of 4.5 μM. Results are the means of two separate determinations which varied by less than 20%.

centration of homofolate (1.0 nM) which inhibited cell growth by a similar extent (see Fig. 1). At a higher concentration of homofolate (20 nM), the reduction in [^{3}H]folate uptake was 90%. Total uptake in control cells without homofolate (3.62 pmol/mg protein) was substantially higher than the amount of binder (0.23 pmol/mg protein) in these cells [4], suggesting that the binding protein had recycled at least 15 times during the 72 hr of cell growth.

Growth response of L1210/JT-1 cells to methotrexate. The relatively weak binding of methotrexate by the folate binding protein suggested that cells utilizing the folate binding protein system to retrieve folate compounds from the medium should be relatively resistant to methotrexate. Growth analysis in 96-well plates showed that L1210/JT-1 cells exhibited an increase in resistance to methotrexate relative to the parental cells, but the degree of resistance (2-fold) was relatively small. When each cell line was

Table 2. Uptake of [^{3}H]folate by L1210/JT-1 cells in culture at various concentrations of homofolate

Cell line	[^{3}H]Folate concentration (nM)	Homofolate concentration (nM)	[^{3}H]Folate uptake (pmol/mg protein)
L1210/JT-1	1.0	0.0	3.62
L1210/JT-1	1.0	1.0	1.74
L1210/JT-1	1.0	20.0	0.35

L1210/JT-1 cells were grown at 37° from an inoculum of 50,000/mL in duplicate 100-mL portions of folate-free RPMI 1640 medium containing 2.5% folate-depleted fetal bovine serum, antibiotics 1.0 nM [^{3}H]folate (sp. act. 10^6 cpm/nmol), and the indicated concentration of homofolate. After 72 hr, cells were harvested by centrifugation, washed twice with HBS, and evaluated for radioactivity and protein. Results are the means of two separate determinations which varied by less than 20%.

grown at 500 nM folate, growth inhibition of L1210/JT-1 cells by 50% occurred at a methotrexate concentration of 23 ± 4 nM (N = 3), whereas growth of the parental cells was decreased by 50% at 12 ± 3 nM (N = 3) methotrexate. A similar 2-fold decrease in methotrexate transport via the reduced-folate transport system was also observed in L1210/JT-1 cells [4], suggesting that the lower sensitivity of L1210/JT-1 cells to methotrexate resulted from the lower activity of the reduced-folate carrier system.

DISCUSSION

L1210/JT-1 cells had been shown previously to grow in the presence of nanomolar concentrations of folate, and the basis for this growth response was attributed to the coordinate up-regulation of a high-affinity folate binding protein and to the mediation by this protein of folate internalization [4]. In the present study, homofolate was found to inhibit coordinately both substrate binding to this protein and cell growth, further supporting a role for the binding protein in folate internalization. The most likely explanation for growth inhibition at low concentrations of folate (see Fig. 1A) is that homofolate interferes with folate internalization. The high affinity of the binding protein for homofolate permits binding sites which otherwise would be occupied by folate to contain bound homofolate, and this would result in a decline in the ability to accumulate folate. Since L1210/JT-1 cells growing at 1.0 nM folate remain partially folate deficient [4], any decrease in ability to accumulate folate should cause a proportional reduction in growth. Direct measurements confirmed that L1210/JT-1 cells accumulate much lower amounts of folate in the presence of homofolate (Table 2). A similar competition of homofolate with sites on the binding protein could also explain the ability of of homofolate to inhibit the growth of L1210/JT-1 cells in the presence of limiting concentrations of 5-formyltetrahydrofolate (Fig. 2). A portion of the growth inhibition could have also resulted from the antifolate effects of internalized homofolate, but a substantial contribution from this mechanism would not appear likely since previous studies had shown that relatively high concentrations of homofolate are required to disrupt folate coenzyme function [12]. Consistent with these prior findings, parental L1210 cells grown at limiting concentrations of folate (Fig. 1A) or 5-formyltetrahydrofolate (Fig. 2A) were inhibited to only a small extent by a large excess of homofolate. The low response to homofolate appears to result from the relatively poor ability of cells to convert homofolates to polyglutamate forms [17, 18]. Internalization of homofolate in parental L1210 cells should have occurred via the reduced-folate carrier since this system can accommodate a relatively broad spectrum of divalent anions [23]. But the extent of homofolate transport would be low since homofolate is bound by the reduced-folate carrier protein with a low affinity (Table 1).

The development of resistance to methotrexate via alterations in transport depends on the specificity of available transport routes for folate compounds. Hence, systems with a low capacity for transporting methotrexate relative to folate compounds would afford a natural cellular resistance to methotrexate. The high-affinity folate binding protein system of L1210/JT-1 cells represents a system with these inherent properties. The relatively poor binding [4] and transport [24] of methotrexate via this binding protein would favor the uptake of folate compounds versus methotrexate. Thus, cells which contain both a high-affinity folate binding protein and an efficient transport system for methotrexate (such as the reduced-folate carrier system of L1210 cells) could develop resistance to methotrexate by down-regulating the more effective methotrexate uptake route. The predicted resistance of these cells to methotrexate would be high but has not been measured directly in L1210 cells, although a subline of human CCRF-CEM cells with a greatly impaired reduced-folate carrier system, an elevated high-affinity folate binding protein, and an unaltered level of dihydrofolate reductase was shown to be 200-fold resistant to methotrexate [25]. The present results suggest further that cells relying solely on the high-affinity folate binding protein system for intracellular folates would have a high sensitivity to homofolate and hence should be killed by this folate analog. Likewise, it should be possible to prevent the appearance of this form of methotrexate resistance by employing homofolate in combination with metho-

trexate, and the isolation of mutant cells with these transport characteristics should be facilitated by screening methotrexate-resistant cells for homofolate sensitivity.

Recent studies have revealed that L1210 cells contain an additional transport system for folate compounds which has not been reported previously [26]. This system is energy dependent, accommodates folate, 5-formyltetrahydrofolate, and methotrexate with K_t values in the range of 2 to 10 μM, and is activated 10-fold by a reduction in pH from 7.4 to 6.2. The contribution of this second system to total methotrexate influx in wild-type L1210 cells is 1–2% of the total at pH 7.4, with the remainder proceeding via the reduced-folate carrier system. In contrast, folate transport in L1210 cells, when measured at a pH (6.8) representative of cells in culture, occurs about equally between this alternative route and the reduced-folate carrier. The presence of this alternate pH-dependent route was also shown to explain the nearly normal growth response to folate by L1210/R81 cells [26], which are resistant to methotrexate due to a loss of the reduced-folate carrier system [26, 27]. L1210/R81 cells do not contain elevated levels of high-affinity folate binding protein [26]. Retension of cell growth on folate has also been observed with other methotrexate-resistant cell lines with diminished methotrexate transport via the reduced-folate carrier system [3, 27–29]. Folate acquisition in the latter cells may be explained by the utilization of the existing pH-dependent folate transport system or by the presence of elevated levels of a high-affinity folate binding protein.

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