A Novel Class of Genetic Variants of the L1210 Cell Up-regulated for Folate Analogue Transport Inward

ISOLATION, CHARACTERIZATION, AND DEGREE OF METABOLIC INSTABILITY OF THE SYSTEM*

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We have isolated stable variants of the L1210 cell exhibiting increased transport inward of the folate analog, methotrexate. These variants show 3- to 14-fold increases in [3H]methotrexate influx compared to parental cells but are unaltered for [3H]methotrexate efflux. This increased influx in each variant is quantitatively reflected in corresponding elevations in intracellular exchangeable levels of drug at steady state, but there is no alteration in membrane potential. The increases in influx are associated with increased values for influx V\textsubscript{max} for a system normally transporting reduced folates and the same increase in the amount of a specific binding component at the cell surface. Otherwise, values for influx K\textsubscript{m} and specificity for various folate structures are unchanged. This alteration in [3H]methotrexate influx is biochemically and genetically stable, since it is expressed in isolated plasma membrane vesicles and is retained during growth in nonselective medium. Following addition of cycloheximide, the same rate of decay of this transport activity (t\textsubscript{1/2} = 126 ± 24 to 137 ± 26 min) was shown for parental and variant cells. From these results we conclude that turnover of this transport property occurs in these cells which is genetically regulated. Also, the elevated transport activity inward for this folate analog in these variant cells is probably the result of a genetic alteration up-regulating the rate of synthesis of the "putative" carrier protein itself. The absence of any effect on efflux of [3H]methotrexate in these variants in the face of evidence for increased synthesis of the carrier protein for the system mediating influx of this folate analog is construed as further evidence for the nonidentity of systems mediating each flux that we proposed on the basis of earlier kinetic studies.

Methotrexate and other folate analogs are accumulated in tumor cells by a transport system (reviewed in Refs. 1 and 2) normally mediating accumulation from plasma of the coenzyme, 5-methyltetrahydrofolate. Since transport of folate analogs is a determining factor in their chemotherapeutic effectiveness (reviewed in Ref. 2), knowledge as to the properties of this system is not only of biochemical interest but has pharmacologic significance as well. To date, biochemical studies of this specific membrane property, particularly those which focus on the putative carrier component, have been hampered by the fact that it appears to exist in the plasma membrane at very low levels (3) when compared to systems mediating transport of many other nutrients (4, 5). For this reason we have sought methods for the isolation of tumor cell variants which are "up-regulated" for this specific membrane property and, hopefully, have elevated levels of the putative carrier in the plasma membrane. The availability of this type of mutant phenotype would also be of value along with phenotypes exhibiting down-regulation of transport in delineating the mechanism of reduced folate transport in these tumor cells. Similar genetic approaches have been successfully applied (6–11) to the study of mechanisms transporting amino acids and cations in mammalian cells.

We now report on the isolation and properties of three variants of the L1210 leukemic cell which show modest to substantial elevation in folate analog transport inward. These increases are associated with increased values for influx V\textsubscript{max} but unchanged values for influx K\textsubscript{m} or specificity for various folate compounds, and the same increase in the amount of a specific binding component at the cell surface. The same kinetic alteration is fully expressed in isolated plasma membrane vesicles and appears to be genetically stable. Data was also obtained showing that this property was metabolically unstable with the same rate of decay of this transport activity in parental and variant cells treated with cycloheximide. These results suggest that the elevated transport activity inward for this folate analog in these variant cells is a result of an increased rate of synthesis of the "putative" carrier itself.

EXPERIMENTAL PROCEDURES

Rationale for Variant Cell Selection—This was based upon our earlier (12, 13) studies of methotrexate-resistant L1210 cells which were reduced in folate compound transport inward and collateral sensitivity to the lipophilic nonclassical antifolate, metoprine. These variants exhibited a 5-fold reduction in influx V\textsubscript{max} for methotrexate and reduced folate compounds which partially accounted for their resistance. Although this relatively finite decrease in influx V\textsubscript{max} for reduced folate compounds was not growth limiting, when such a compound was used as the sole "folate" source, it was growth limiting in the presence of metoprine. This agent, like methotrexate (reviewed in Ref. 14), blocks regeneration of Hzfolate\textsuperscript{1} from H,folate and, thus, in its presence must be replenished exogenousy. Also, since metoprine is highly lipophilic, its entry into tumor cells, in contrast to folate analogs, requires only simple diffusion (15). This deficiency in reduced folate transport served to explain (13) the increased sensitivity to metoprine, since increased sensitivity of these variants was not observed (13) when only folic acid was in the growth medium (this folate has (16, 17) a more preferred route of entry into L1210 cells).

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1 The abbreviations used are: Hz, dihydro; H, tetrahydro; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
We reasoned, therefore, that it might be possible to obtain variants with increased capacity for transport of reduced folate compounds by selecting for metoprine resistance with 5-formyl-H4folate as the sole folate source. This phenotype could be readily identified by examining for increased sensitivity to methotrexate. We also reasoned that the same variant phenotype might be selected during growth in suboptimum 5-formyl-H4folate. Both procedures proved adequate for the derivation of the desired variants.

**Isolation Procedures**—(a) By methods described previously (18), L1210 cells were grown in RPMI medium supplemented with dialyzed calf serum and the minimum concentration of 5-formyl-H4folate required for maximum growth. Cell suspensions were transferred to the same medium containing an IC50 concentration of metoprine. After cell density approached stationary phase of growth, cells were transferred again in this medium. This was repeated until the doubling time of the culture was similar to controls. Cells from drug medium were then cultured by resuspending in drug medium containing 0.3% agar. After 7-10 days of incubation at 37 °C colonies were picked and screened for increased sensitivity to methotrexate using 2.2 μM folic acid as the sole folate source. Subcultures showing increased sensitivity were examined (2) for methotrexate transport. (b) Cell suspensions grown in the same medium as above were transferred to medium of 5-formyl-dialyzed calf serum and 5-formyltetrahydrofolic acid at the EC50 concentration. After the cell number approached stationary phase, additional transfers were made until the doubling time was similar to controls. Cloning and screening of subcultures was carried out as described above.

**Variants with Increased Folate Antagonists**—A rapid sampling procedure modified (19) from Plagemann and co-workers (20) was employed during these studies for processing of samples by radioactive scintillation counting. This procedure employed a correction for extracellular radioactivity (17) which yielded the intracellular radioactivity. Values for intracellular water were derived (17) for all of the cell types used in this study and varied from 2.9 ± 0.3 to 3.5 ± 0.4 ml/g dry wt in agreement with values also determined from wet and dry weight determinations (2). The derivation of values for membrane potential has been described (2, 21). This was derived from measurements of cell chloride which varied only from 2.69 ± 22 to 2.96 ± 51 μmol/g dry weight, among the cell types studied. Data derived during transport experiments were expressed as nanomoles/g dry wt, in accordance with conventions established in our earlier studies (2). Data for intracellular drug concentration are expressed as values for exchangeable drug, that is unbound to dihydrofolate reductase. Intraacellular levels of this enzyme were determined as described earlier (2).

Transport experiments were carried out at 37 °C with cell suspensions (2-3 × 107 cells/ml) prepared in buffer-salts solution containing 107 mM NaCl, 20 mM Tris-HCl, 36.2 mM NaHCO3, 5.3 mM KCl, 1.9 mM CaCl2, 1 mM MgCl2 with 7 mM d-glucose at pH 7.4. In some experiments, we used a buffer containing 150 mM Hepes (Sigma) and 2 mM MgCl2. The pH was adjusted to 7.4 with 66.2 mM KOH. Concentrations employed (2) for these determinations ensured measurements of unidirectional influx and efflux of [3H]methotrexate. Experimental procedures used to derive values for influx Vmax, influx Km and efflux rate constant have been described in detail (2).

**Binding Assay**—In a modification of that described by Henderson et al. (3), the assay mixture used contained 3-5 × 107 L1210 cells and [14C]aminopterin in 150 mM Hepes buffer plus 2 mM MgCl2 adjusted to pH 7.4 with 66.2 mM KOH. Following incubation for 5 min at 0-4 °C samples were centrifuged in an Eppendorf microcentrifuge at 12,000 × g for 1 min (19). Supernatant was removed by suction and residual fluid removed with cotton swabs. Pellets were then resuspended in 25 μl with 66.2 mM KOH. Concentrations employed (2) for these determinations ensured measurements of unidirectional influx and efflux of [3H]methotrexate. Experimental procedures used to derive values for influx Vmax, influx Km and efflux rate constant have been described in detail (2).

**Growth Inhibitory Effects of Methotrexate and Metoprine against Parental and Variant L1210 Cells**—Time courses for [3H]methotrexate accumulation at 37 °C in variant L1210 cells exhibited characteristics similar to those already reported (2, 12, 13) from our laboratory for parental L1210 cells. Representative data for one variant (R69) and the parental cell type following exposure to 1 μM [3H]methotrexate are shown in Fig. 1A. Following an initial linear phase of uptake, intracellular accumulation was characterized by an exponential approach to steady state (2-50 min). Measured values of t1/2 were in the range of 3-4 min, and accumulation is described by a function of the form

\[ c(t) = C_0(1 - e^{-2.7t}) \]

where C0 is the steady state, Km is the rate constant for efflux of drug, and t0 is the onset of free drug accumulation. In this variant (R69) and the other variants (data not shown), initial uptake and levels of accumulation of exchangeable drug (not bound to dihydrofolate reductase) at steady state were greater than in the parental cell type (Fig. 1A). In contrast to the

**RESULTS**

**Growth Inhibition of Parental and Variant L1210 Cells by Folate Antagonists**—Three variant cell types were isolated by the selective procedure described above. The sensitivity of these variants compared to parental L1210 cells to growth inhibition by methotrexate and methotrexate are shown in Table I. The variants (R26, R69, and R82) show 3- to 26-fold increases in resistance to methotrexate compared to parental L1210 cells. Conversely, their sensitivity to methotrexate was increased by 3- to 7-fold. It should be noted that both R69 and R82 cells exhibit 2-fold elevation in the intracellular target, dihydrofolate reductase, which would account for some of the increased resistance to methotrexate and tend to offset the increased sensitivity to methotrexate due to transport alterations. For comparison, we also show data for a cell line (R1) shown in our earlier (12) studies to have a reduced Vmax for methotrexate influx. This cell line shows increased sensitivity to metoprine but decreased sensitivity to methotrexate.

**Characteristics of Methotrexate Transport in Parental and Variant L1210 Cells**—Time courses for [3H]methotrexate accumulation at 37 °C in variant L1210 cells exhibited characteristics similar to those already reported (2, 12, 13) from our laboratory for parental L1210 cells. Representative data for one variant (R69) and the parental cell type following exposure to 1 μM [3H]methotrexate are shown in Fig. 1A. Following an initial linear phase of uptake, intracellular accumulation was characterized by an exponential approach to steady state (2-50 min). Measured values of t1/2 were in the range of 3-4 min, and accumulation is described by a function of the form

\[ c(t) = C_0(1 - e^{-3.5t}) \]

where C0 is the steady state, Km is the rate constant for efflux of drug, and t0 is the onset of free drug accumulation. In this variant (R69) and the other variants (data not shown), initial uptake and levels of accumulation of exchangeable drug (not bound to dihydrofolate reductase) at steady state were greater than in the parental cell type (Fig. 1A). In contrast to the

**TABLE I**

| Cell line | Dihydrofolate reductase level | Metoprine IC50 | Methotrexate IC50 | Ratio (metoprine/ methotrexate) |
|-----------|-------------------------------|----------------|-------------------|---------------------------------|
| L1210     | 3.75 ± 0.5                    | 16.3 ± 2.2     | 2.82 ± 0.3        | 6.0                             |
| L1210/R1  | 3.47 ± 0.4                    | 3.5 ± 0.4      | 29.7 ± 2.7        | 0.178                           |
| L1210/R26 | 3.59 ± 0.4                    | 44.2 ± 5.3     | 1.13 ± 0.2        | 35.9                            |
| L1210/R69 | 7.34 ± 0.9                    | 259 ± 27       | 0.71 ± 0.8        | 38.5                            |
| L1210/R82 | 7.52 ± 0.7                    | 428 ± 49       | 0.42 ± 0.5        | 102.0                           |

*Values given are averages of 3 determinations ± S.E. of the mean.*
influx in each case was characterized by a single saturable component even though relative influx overall varied substantially between variant and parental cell types. The kinetic properties of mediated \([{}^{3}H]\)methotrexate transport in these various cell types are compared in Table II. Values for influx \(K_{m}\) showed small differences among parental and variant cell types. However, values for influx \(V_{\text{max}}\) derived with all of the variant cell types were greater than that derived for parental L1210 cells by 3- to 15-fold. Values for the time constant \((K_{t})\) to achieve steady state derived for all of the cell types were essentially identical and equal to the same values derived for efflux \((K_{t}^{e})\). Measured values for exchangeable levels at steady state (as in Fig. 1A) were within 10% of those calculated using the following empirical equation,

\[
C_{T} = \frac{V_{\text{max}}[\text{drug}]_{0}}{(K_{m} + [\text{drug}]_{0})K_{t}}
\]

(2)

and the values derived for each constant shown in Table II. No expression for a diffusion component is needed in this equation, since this component at physiological drug concentrations is negligible (2). The transport properties of these variant cells were the same (data not shown) after growth in culture during several transplant generations in the absence of drug and with folic acid as the sole folate source.

In other studies, we examined the structural specificity of the system-mediating influx of \([{}^{3}H]\)methotrexate in parental and variant L1210 cells. Specificity of influx for various structures were determined from competition experiments measuring the inhibition of mediated \([{}^{3}H]\)methotrexate entry. From the results of these experiments (Table III) it can be seen that the system mediating \([{}^{3}H]\)methotrexate influx exhibits similar specificity in both parental and variant cell types examined. In each case, the affinity of the system, as inferred from the individual values for influx \(K_{i}\), was greatest for the folate analogs, aminopterin and 10-deaza-aminopterin, and lowest for folic acid by 2 orders of magnitude. Values for \(K_{i}\) were in the order, aminopterin \(\equiv\) 10-deaza-aminopterin \(<\) 5-formyltetrahydrofolate \(<\) methotrexate \(<\) folic acid. For methotrexate, values for influx \(K_{i}\) \(\equiv\) values for influx \(K_{m}\).

Specific Surface Membrane Binding of \([{}^{3}H]\)Aminopterin by Parental and Variant L1210 Cells—The various cell types were examined for their ability to specifically bind \([{}^{3}H]\)aminopterin at the cell surface. Data derived from this binding assay appears (3, 26) to be a measure of the putative carrier component of the reduced folate transport system, since this binding exhibits (3) structural specificity similar to influx and is decreased (26) in transport-deficient cell lines. Data is shown in Fig. 2 for the binding of this analog by parental and R82 cells in the absence of other anions (Hepes-Mg-sucrose buffer). A larger specific binding component on R82 cells compared to parental L1210 cells was delineated by the addition of nonradio labeled aminopterin. From a double-recip-

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**Table II**

**Kinetic properties of mediated transport of \([{}^{3}H]\)methotrexate by parental and variant L1210 cells**

| Cell type       | Method of selection | Influx | Efflux |
|-----------------|---------------------|--------|--------|
|                 |                     | \(K_{m}\) | \(V_{\text{max}}\) | \(K_{t}\) | \(K_{t}^{e}\) |
|                 | \(\mu M\)           | \(\text{nmol/min/g. dry wt}\) | \(\text{min}^{-1}\) | \(\text{min}^{-1}\) |
| L1210           | A                   | 4.65 ± 1.2 | 5.45 ± 0.6 | 0.172 ± 0.01 | 0.161 ± 0.02 |
| L1210/R26       | B                   | 5.35 ± 0.6 | 12.1 ± 1.1 | 0.178 ± 0.02 | 0.163 ± 0.03 |
| L1210/R69       | B                   | 5.33 ± 0.8 | 46.1 ± 9.1 | 0.162 ± 0.02 | 0.184 ± 0.01 |
| L1210/R82       | A and B             | 5.59 ± 0.9 | 78.5 ± 9.8 | 0.179 ± 0.05 | 0.178 ± 0.02 |
TABLE III

Structural specificity of folate compound transport in variant and parental L1210 cells

Data were derived during experiments measuring inhibition of [³H]methotrexate influx. Methodology and data analysis were described earlier (2) and in the text or legend of Fig. 1. Numerical values are averages of 3 separate experiments ± S.E.

| Compound          | L1210     | L1210/R26 | L1210/R82 |
|-------------------|-----------|-----------|-----------|
|                   | µM        | µM        | µM        |
| Aminopterin       | 1.31 ± 0.2| 1.72 ± 0.3| 1.80 ± 0.2|
| Methotrexate      | 4.83 ± 0.7| 4.94 ± 0.6| 5.18 ± 0.8|
| 10-Deaza-aminopterin| 1.42 ± 0.3| 1.54 ± 0.4| 1.58 ± 0.4|
| Folic acid        | 2.93 ± 0.4| 1.92 ± 0.3| 2.78 ± 0.4|
| 5-CHO-H₄folate    |           |           |           |

*Expressed as the natural diastereoisomer in the racemic mixture employed.

FIG. 2. Binding of [³H]aminopterin by parental and variant L1210 cells in the presence and absence of excess nonradioactive aminopterin. Cells were incubated with varying concentrations of [³H]aminopterin for 5 min at 0°C in the presence or absence of 500 µM aminopterin. Values shown are averages of 3-5 determinations ± S.E. Additional details are given in the text.

FIG. 3. Time courses for accumulation of [³H]methotrexate by vesicles prepared from parental and variant (R82) L1210 cells. Aliquots of vesicle preparations were equilibrated for 30 min at 37°C in 50 mM potassium phosphate, 100 mM NaCl, and 150 mM sucrose at pH 7.4 after which 2 pM [³H]methotrexate was added. Aliquots of vesicle suspension were removed at various intervals during incubation at 37°C until equilibration was achieved and processed for radioactive counting. Additional details are given in the text. Standard error of the mean did not exceed ±13%. Values shown are averages of 3 separate experiments.

FIG. 4. Decay of [³H]methotrexate influx in L1210 cells treated with cycloheximide. Protein synthesis was measured from the rate of [³H]leucine incorporation (31) into cellular acid-insoluble precipitates and found to be nearly completely inhibited 30 min after adding 5 mg/ml of cycloheximide to the culture medium. The concentration of [³H]methotrexate was 2 µM. Additional details are provided in the text. Values shown are averages of 3-5 experiments. S.E. was less than ±13%.

*Excluded as the natural diastereoisomer in the racemic mixture employed.
Methotrexate Transport in L1210 Cell Variants

The method of selection employed during these studies appear to provide a means for the isolation of the desired genetic variants of the L1210 cell with elevated transport of reduced folates and folate analogs. This elevated membrane activity could be attributed to a transport system which was a counterpart of the same system in parental cells, since both systems exhibited the same specificity for various folate compounds. Although genetic analysis has not been carried out with these variants, from the manner of their isolation and the stability of the variant property in the absence of selective pressure, they are assumed to have originated by alteration of some genomic element in parental L1210 cells. The phenotype characterized in these studies could have originated in one of a number of ways. The explanation, most likely in view of the data, is that they arose form an alteration of some regulatory component which constitutively controls the rate of synthesis of the putative transport protein. We found that the elevation in influx \( V_{max} \) characteristic of these variants is associated with a proportional increase in surface membrane content of a specific binding component. Also, both the influx \( K_m \) and the binding \( K_0 \) were essentially unaltered in these variants, and the system elevated in variant cells exhibited the same specificity for various folate compounds as does the system in parental cells. The full extent of expression of this elevated transport property in plasma membrane vesicles tends to eliminate the involvement of altered cytosol-associated energy metabolism and shows that this increased transport capacity is fixed in the isolated membrane itself. Since directional asymmetry for transport by intact L1210 cells is not maintained (17, 23, 24, 30) in membrane vesicles, it is unlikely that greater asymmetry in intact variant cells is the reason for this increased transport activity. Finally, since we have shown that the rate at which influx of \([3H]methylotrexate\) decays is the same in both variant and parental cells, the increased membrane content of this system can probably be explained by increased rate of synthesis of some protein component.

Metabolic turnover of \([3H]methylotrexate\) influx capacity in cells treated with cycloheximide—We also sought information on the basis for the apparent increase in plasma membrane component of the reduced folate transport system in these variant cell types suggested by the results described above. If this system was metabolically unstable, then depending upon its rate of degradation in parental and variant cells, the increase in membrane content of this system could be attributed to either increased synthesis or decreased degradation. To determine to this, we measured initial influx of \([3H]methylotrexate\) in parental L1210 cells during growth in cell culture with 5 mg/ml of cycloheximide. At this concentration of cycloheximide, protein synthesis, as measured by \([3H]leucine\) incorporation into acid-insoluble cellular material, was inhibited nearly 100% (data not shown). Initial influx was reduced within 30 min after cycloheximide treatment, and further reduction occurred during the 5-h period in which measurements were made. An analysis of this data is shown in Fig. 4, where it can be seen that influx exhibited an exponential decline with a decay time \((t_{1/2})\) of slightly above 2 h. During this period only minimal effects were seen on cell viability as determined by Trypan blue exclusion or clonogenic assays. Cell viability decreased from 92–95% to 90–92%. The same experiment was also carried out with cultures of cell transport properties in human malignant cells, as the variety of the system is analogous to that described previously (21, 27) in distribution of carrier at each membrane surface. It was of interest, therefore, to determine to what extent the altered properties in these variants were expressed in isolated plasma membranes. To do this, we employed methodology for preparing plasma membrane vesicles from the various cell types under study. In these membrane preparations, and unlike intact L1210 cells (21, 27–29), transport of folate compounds is equilibrating (17, 23, 24) and shows directional symmetry (30). Time course for accumulation of \([3H]methylotrexate\) at an external concentration of 2 \(\mu\)M at 37°C by vesicles from parental and R82 variant L1210 cells is shown in Fig. 3. At this concentration and at all others employed (data not shown) the initial 30–60 s of accumulation is linear with time. Initial influx of drug by the vesicles prepared from variant cells was substantially greater than in vesicles derived from parental cells, although steady-state levels ultimately achieved were approximately the same. A kinetic analysis of the data obtained for initial influx of \([3H]methylotrexate\) at various external concentrations revealed an increase of approximately 12-fold of influx \( V_{max} \) (parental cells = 8.21 ± 1.1 pmol/min/mg of proteins; R82 cells = 98.4 ± 13 pmol/min/mg of protein), while values for influx \( K_m \) (4.33 ± 0.5 to 4.61 ± 0.6) were essentially unchanged.

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In contrast to that seen with intact L1210 cells, initial efflux of \([3H]methylotrexate\) from R82-derived vesicles was also 10- to 12-fold greater than for parentally derived vesicles. This is consistent with a simple carrier model for facilitated diffusion and bidirectional flux proposed for these vesicle preparations on the basis of our earlier studies (21, 27).
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facilitate the isolation of this component. This could then lead the way to the development of cloning procedures necessary for probing the molecular genetics of this system.

Finally, we believe the biochemical data derived in these studies is further evidence for the notion of separate systems mediating influx and efflux of these analogs in L1210 cells which was proposed on the basis of our earlier kinetic studies (21, 27). Evidence derived for an increased rate of synthesis of a specific binding protein in these transport-elevated variants would appear to make it less likely that the absence of an effect on efflux was a kinetic anomaly.

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