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Inhibition of methionine uptake by methotrexate in mouse leukemia L1210 cells*

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Summary. Methionine-auxotrophic L1210 cells were used to study the effect of methotrexate (MTX) on methionine uptake and metabolism. MTX was shown to inhibit amino acid transport systems and cause a decrease of methionine uptake into L1210 cells. Conversely, a nonmetabolizable amino acid analogue reduced MTX uptake into L1210 cells. MTX also blocked the transfer of the beta carbon from serine into methionine. Therefore, methionine deprivation may be an additional mechanism of action for MTX in methionine-auxotrophic tumor cells.

Introduction

Methionine, an essential amino acid, has been known to regulate folate biosynthesis [8, 9] and is an absolute requirement for cell growth in L1210 cells [8]. Since methotrexate (MTX) acts as a potent inhibitor of folate biosynthesis, the role of MTX on both methionine metabolism and transport into L1210 cells was examined.

Amino acids are transported into mammalian cells by three transport systems: the A, L, and ASC systems [3]. Essential amino acids are concentrated primarily by the ASC system in most cells in culture [4, 10, 18]. Inhibition of this transport system by MTX may contribute to its cytotoxicity. Differences in substrate specificity of the ASC system have also been demonstrated between normal rat hepatocytes and methionine-auxotrophic hepatoma cells (HTC) [6].

Mitogen-stimulated human lymphocytes and human leukemic K562 cells are also auxotrophic for methionine [9], and the uptake of methionine into these cells has been shown to be inhibited by MTX [14, 15]. Drug-membrane interactions in L1210 cells are not unique; homofolate [13], cisplatin [16, 17], and adriamycin [20] have also been demonstrated to influence cell growth at the membrane [7]. This paper presents evidence that MTX can inhibit methionine uptake and metabolism in L1210 cells.

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Materials and methods

Chemicals. L-(1-^{14}C)-Methionine (50 mCi/mmol), (3-^{14}C)-serine (53 mCi/mmol), and (3', 5', 7-^{3}H)-methotrexate (205 mCi/mmol) were purchased from Amersham, Arlington Heights, Ill. (1-^{14}C)-Aminoisobutyric acid (AIB; 51.6 mCi/mmol) was purchased from New England Nuclear, Boston, Mass. MTX was obtained from Lederle Laboratoires, Pearl River, New York.

Cells and growth medium. Cultures were established from frozen mycoplasma-free stocks at 3-month intervals. Stock suspension cultures of L1210 were grown in Fischer's medium as previously described [14]. Soft agar cloning was carried out as previously described [17].

Incubation medium for amino acid transport studies. The incubation medium (IM-1) for determining sodium-dependent amino acid transport was Earle's balanced salt solution (GIBCO). The incubation medium (IM-2) for determining sodium-independent transport was Earle's balanced salt solution in which 116 m*M* choline chloride was substituted for 116 m*M* sodium chloride [16].

Transport measurement. For each transport measurement, cells were preincubated in the appropriate medium for 15 min at 37 °C [16]. The cells were preincubated with MTX for 2 min prior to the addition of labeled substrate. The addition of radioactivity marked the zero timepoint [16]. At timed intervals, 200-µl samples were withdrawn and immediately separated from the incubation medium by centrifugation through a silicone-mineral oil gradient into perchloric acid [16].

The data in the figures represent the means of three experiments. In these representative experiments, the standard error was less than 10% of the mean. Background was subtracted from these results by calculating the zero timepoint, defined as the nonspecific binding component.

Because of the large differences in specific activities and molar concentrations between the amino acids and MTX, a modification in the transport and washing procedure was required. The uptake of MTX was determined by a previously published method [12]. In the competition experiments, unlabeled AIB was added to the reaction mixture simultaneously labeled MTX. The data in Fig. 5 represent the mean of three experiments. In these representative experiments, the standard error was less than 10% of the mean.

Results

Methionine requirements for optimal L1210 cell growth

Fischer's media without methionine was cytotoxic to the L1210 cells. Incubation of L1210 cells in Fischer's medium lacking methionine for 1 h resulted in only 40% viable cells as measured by soft agar cloning after 10 days or by outgrowth studies after 48 h (Fig. 1). The addition of methionine to the media even after only 1 h did not reverse this cytotoxicity.

L1210 cells were incubated with 250 μ*M* (3-^{14}C)-serine in the presence or absence of MTX. The L1210 cells metabolized 26% of the cell-associated, labeled serine into acid-insoluble material in 15 min, while 29% of the acid-soluble label was metabolized into methionine. MTX completely inhibited the transfer of the beta carbon of serine into the pool of methionine in 15 min as measured by paper chromatography.

Amino acid uptake

The initial uptake of AIB (500 μ*M*) was linear for 2 min (Fig. 2). AIB concentration was at least three times higher in the presence of sodium chloride than choline chloride (Fig. 2). Methionine (500 μ*M*) uptake was rapid for 30 s (Fig. 3) and continued for at least 10 min. Methionine intermediates (14%) were detected following a 2-min exposure of L1210 cells to methionine. The possible breakdown products as determined by APLC which had the retention times of methionine sulfoxide and methionine sulfone, did not significantly inhibit methionine uptake.

MTX effects on amino acid transport

As shown in Figs. 2 and 3, MTX did not affect the sodium-independent uptake of AIB and methionine. However, MTX did reduce sodium-dependent AIB and methionine uptake at concentrations of 10 and 25 μ*M*. The initial sodium-dependent uptake of AIB was inhibited 53.4% by 10 μ*M* MTX, while the methionine uptake was lowered

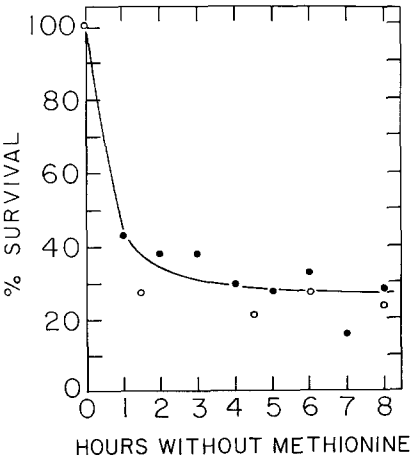

Fig. 1. Methionine requirements for L1210 cell growth. L1210 cells were resuspended in Fischer's medium depleted of methionine for 1–8 h, then cloned in complete Fischer's medium by soft agar and counted 10 days later (●) or grown and the cell number determined in complete Fischer's medium 48 h later (o). Cell growth in complete medium had a cloning efficiency of 90% or greater and was standardized as 100%

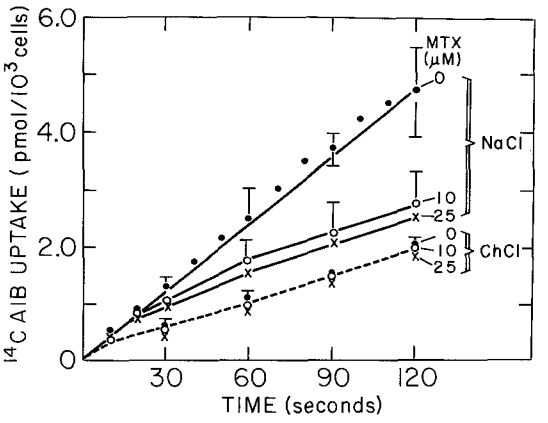

Fig. 2. AIB uptake in L1210S cells in the presence or absence of MTX. Initial AIB (500 μ*M*) uptake was measured in the presence of sodium chloride (IM-1, ●———●) or choline chloride (IM-2, ●———●) without MTX. Cells were preincubated for 1 min in the presence of 10 μ*M* (o———o) or 25 μ*M* (×———×) MTX. See Methods for details

33%. This same concentration of MTX did not alter the metabolism of methionine significantly as determined by HPLC (data not shown).

AIB uptake was more sensitive to MTX inhibition (0.01 μ*M*) than was methionine uptake, which required a concentration of MTX greater than 0.1 μ*M* for inhibition (Fig. 4). Maximum MTX inhibition of amino acid transport was dependent on a short preincubation period. MTX had no significant influence on sodium-independent amino acid uptake in L1210 cells. Washing the cells free of MTX did not restore their transport properties. Finally, MTX did not alter the efflux of amino acids.

Effect of amino acids on MTX uptake

Methotrexate-sensitive L1210 (L1210S) cells transported MTX at a linear rate for at least 15 min in IM-1 buffer (Fig. 5). During the first 5 min, AIB (500 μ*M*) had no signi-

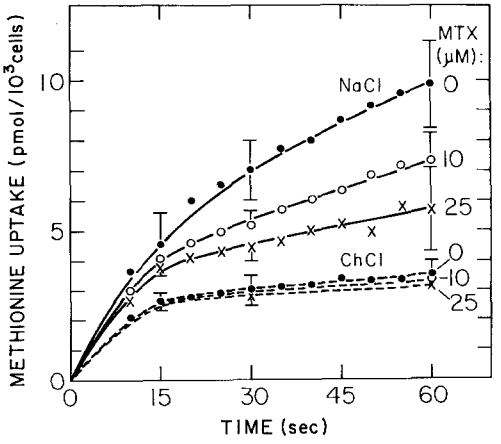

Fig. 3. Methionine uptake in L1210S cells in the presence or absence of MTX. Methionine (500 μ*M*) uptake was measured in the presence of sodium chloride 6IM-1 alone, ●———●) or choline chloride (IM-2, ●———●). Cells were preincubated for 1 min in the presence of 10 μ*M* (O) or 25 μ*M* (×) MTX. See Methods for details

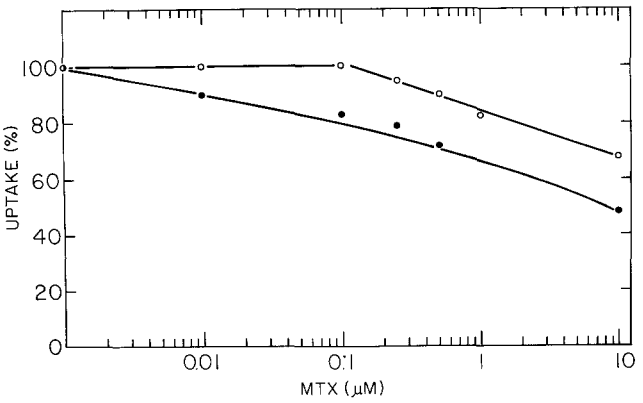

Fig. 4. Concentration-dependent MTX inhibition of amino acid transport. L1210S cells were preincubated with varying concentrations of MTX (0.001–10 μ*M*) for 1 min. AIB (●) and methionine (○) uptake was measured at timed intervals in IM-1 (details described in Methods). The 100% value for AIB was 1 min, and for methionine, the 1-min timepoint in the absence of MTX

ficant influence on the uptake of MTX (25 μ*M*). However, the uptake of MTX after 5 min was partially blocked (40%) by AIB.

Discussion

In L1210 cells, MTX may act not only through inhibition of DNA and purine synthesis, but also through methionine deprivation. MTX causes a perturbation of the folate pools in tumor cells [1, 19], and methionine could be growth-limiting in some of these tumors [8, 9].

In L1210 cells, the metabolism of the beta carbon of serine into methionine is important for cell viability [14]. MTX has been shown to inhibit this pathway in L1210 cells by rapidly decreasing the 5-methyltetrahydrofolate pool and limiting the beta carbon transfer from serine to methionine [19].

Exogenous methionine has been shown to be growth-limiting for L1210 cells. Methionine and other essential amino acids are concentrated by a sodium gradient through the ASC amino acid transport system in tumor cells [2, 18]. This ASC system in tumor cells has transport properties different from those associated with normal cells [6]. In L1210 cells, this transport system has been termed "a sodium dependent leucine preferring system" [21]. AIB, a nonmetabolizable amino acid analog, was used to discriminate amino acid transport from metabolism, especially since AIB and methionine have been shown to share similar transport systems in L1210 cells [16]. MTX inhibition of AIB uptake into L1210 cells would suggest that MTX causes cytotoxicity by inhibiting methionine transport in addition to disrupting intracellular methionine metabolism. In addition, MTX has previously been shown to inhibit methionine influx in human cells [14, 15]. The present study revealed a greater than 30% reduction of sodium-dependent methionine uptake into L1210 cells upon MTX addition.

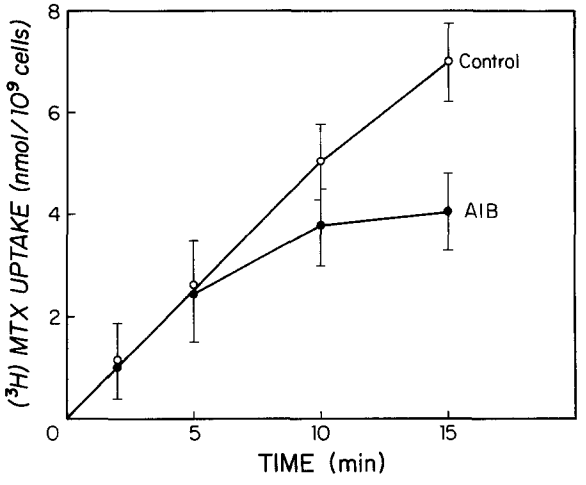

Fig. 5. Labeled MTX uptake in L1210S cells. MTX (10 μ*M*, ○) uptake was measured in the presence of AIB (250 μ*M*, ●). Details described in Methods

Even though the exact inhibitory site for MTX has not yet been elucidated, several possibilities may be considered. For instance, triamterene, a 2, 4-diaminopteridine diuretic, inhibits sodium ion transport across the renal distal tubule membrane [22]. Therefore, it is conceivable that the 2, 4-diamino structure of MTX could either inhibit dihydrofolate reductase or interact at the membrane or at other cellular sites, as well as inhibiting sodium ion transport [1, 2, 5]. Inhibition of methionine uptake by MTX may be due to (a) drug binding to a specific membrane carrier or (b) reduction of the sodium gradient across the plasma membrane necessary for the uptake of amino acids.

In conclusion, MTX cytotoxicity in L1210 cells may occur as a result of methionine deprivation, as seen by its ability to inhibit (a) methionine uptake and (b) serine metabolism to methionine in addition to decreasing DNA and purine synthesis. Therefore the clinical efficacy of MTX may in part depend upon the methionine requirements of the tumor cell compared to the normally proliferating cell.

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