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SHORT COMMUNICATIONS

Defective transport of amethopterin (methotrexate) as a mechanism of resistance to the antimetabolite in L5178Y leukemic cells*

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A MUTANT CLONE (m^t) of murine leukemic cells (L5178Y) has been isolated that requires a relatively high concentration of methotrexate, 56×10^{-8} M, to inhibit the rate of reproduction by 50%; this concentration is about 70 times higher than that required for equivalent inhibition of the sensitive parent clone (*s*). The population of resistant cells was selected by the incubation of sensitive cells at a level of methotrexate ($2{\cdot}0 \times 10^{-8}$ M) sufficient to prevent completely their reproduction in a medium otherwise suitable for cellular reproduction.[1, 2] From the emergent resistant population, "second-step" mutant cells were isolated, by methods previously described;[3] one of these cells provided the resistant clone, m^t. This line has quantitatively retained its level of resistance after more than 500 consecutive doublings in methotrexate-free medium, and is fully resistant to methotrexate therapy in mice.

In order to study the uptake of methotrexate by mammalian cells, a system was devised that permitted removal of the extracellular fluid from the cells. After preincubation of the cells for 5 min at 37° at a density of 2×10^7 per ml of growth medium, methotrexate-^{3}H (423 μc/μmole, 3 to $1{,}000 \times 10^{-8}$ M) was added. The cells were recovered by layering 5-ml aliquots over a 36-mm column of cold 0·25 M sucrose in an Addis tube by centrifugation at about $800 \times g$ in a swinging head for 4 min and removing the medium and sucrose by aspiration. The tube was carefully washed twice with cold, nonradioactive medium without disturbing the packed cells; from these, methotrexate was recovered by the addition of 0·6 ml of 0·05 N HCl, heating for 10 min in a boiling water bath, and centrifugation; an aliquot (0·4 ml) was taken for the determination of radioactivity in a liquid scintillation counter. The volume of packed cells was separately determined by the centrifugation of an aliquot (2·0 ml) in a Hopkins vaccine tube in a swinging head for 4 min at about $800 \times g$. The methotrexate was not significantly removed from the cells during centrifugation through the sucrose, as was demonstrated by comparative studies with a system previously described that does not employ sucrose.[4] In addition, cells that had been collected by centrifugation through sucrose fully retained their ability to transport methotrexate.

At equivalent concentrations of methoxtrexate, the uptake by the sensitive line was 14 times more rapid than in the m^t cells. With methotrexate at levels of from 3 to $1{,}000 \times 10^{-8}$ M, the kinetics of uptake in both lines were otherwise similar; thus, a very rapid association (occurring within 30 sec) was followed by an approximately linear time course that extended over 30 min.

After incubation for 30 to 40 min in the presence of methotrexate, 0·3 to 6×10^{-7} M, the concentration of the antimetabolite within the cells remained approximately constant. In *s* cells the intracellular level, after equilibration, was greater than that in the medium by a factor of 2 to 3, while the concentration in m^t cells was only 0·1 to 0·2 that of the medium at the same levels of methotrexate. When the extracellular radioactive methotrexate was replaced by an equimolar level of nonradioactive drug, a rapid displacement of intracellular radioactive methotrexate was observed; the level of radioactivity fell to 20% of the equilibrium value within 25 min in both lines. Cells suspended in a medium free of methotrexate, or even in a methotrexate-free medium containing an excess of a highly purified preparation of folic acid reductase (1,000 U/ml of medium; 14 μmole product/hr/mg protein, with folic acid as the substrate) lost intracellular methotrexate at a slow rate. The uptake system approached saturation at a concentration of drug of 10^{-5} M in the medium. Cells from either *s* or m^t-lines took up the drug in only small amounts at 0°. These findings suggest that methotrexate was

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located within the cell in a form able to participate in an exchange process. The data do not permit an explanation, at the molecular level, of the difference between the transport mechanisms in the two types of cells. It is possible that a carrier protein, localized in the cell membrane, is altered in the m^t cells.

The folic acid reductase of m^t cells had an unaltered "affinity" for methotrexate, was of equal activity in s and m^t cells, and behaved similarly during fractionation. At 10^{-5} M methotrexate, s cells were capable of taking up 10 times the amount of methotrexate required to titrate the total folic acid reductase activity within the cell. Fractionation of such cells revealed that the methotrexate was only in small part associated with this enzyme; the major fraction was dialyzable, whereas a minor fraction was tightly bound to material without folic acid reductase activity. In addition, the intracellular methotrexate from s cells incubated for a period of 18 hr at a completely inhibitory, but much lower, concentration of the drug (3×10^{-8} M) was recovered as extracellular drug by incubation of the cells for 6 hr in growth medium free of methotrexate. The quantity of methotrexate recovered suggested that the inhibitor can be freed from the folic acid reductase of these cells.

The specificity of the mutagenic events affecting transport in m^t cells was indicated by the observation that the nutritional requirements for very high levels of folic acid (and of thymidine, hypoxanthine, and serine in the presence of inhibitory levels of methotrexate), were unchanged in the m^t cells. In addition, the sensitivity of the m^t cells to selected antimetabolites during reproduction in culture (cytosine arabinoside, 6-azauridine, and 5-fluorodeoxyuridine) was unchanged; furthermore, methotrexate was not significantly inactivated by either line of cells.

The levels of folic acid reductase activity of s and m^t cells was not altered by growth of the cells in the presence of partial or completely inhibitory levels of methotrexate, a finding which suggested that enzyme induction did not occur in either line in response to the drug. Similar findings concerning enzyme induction had been reported for those mutants that become resistant by virtue of an increased level of folic acid reductase activity.[5] However, m^t cells had an increased nutritional requirement for DL-leucovorin. It has also been demonstrated that 10^{-5} M DL-leucovorin inhibits the uptake of 3×10^{-8} M methotrexate by sensitive and resistant cells. Thus, it is possible that the transport mechanism for methotrexate may also be concerned with the entry of other derivatives of folic acid into the cell.

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The subcellular distribution of substance of P and 5-HT in brain

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SEPARATION of the crude mitochondrial fraction (P2) from brain homogenates in a density gradient of 0·32, 0·8 and 1·2 M sucrose solutions produces three subcellular fractions: a light myelin fraction, a nerve ending fraction of intermediate density and a denser mitochondrial fraction.[1] Pharmacological analyses of these fractions show that acetylcholine (ACH)[2, 3] and substance P [2] are mainly located in the nerve ending fraction. However in guinea-pig brain homogenates which contain 1 mM iproniazid, 5-HT is mainly recovered from the mitochondrial and also from the microsomal fractions.[2] Preliminary experiments on rat brain homogenates, which did not contain iproniazid, indicated that 5-HT