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PRELIMINARY COMMUNICATIONS

EXTRACELLULAR RECOVERY OF METHOTREXATE-POLYGLUTAMATES FOLLOWING EFFLUX FROM L1210 LEUKEMIA CELLS

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The intracellular biosynthesis of poly-γ-glutamyl metabolites of methotrexate (MTX) has been demonstrated [1-3] in a variety of mammalian tissues. Also, these polyglutamates appear to be [2,4] as effective as MTX as inhibitors of dihydrofolate reductase. While some reports [5,6] have suggested that these polyglutamates are transported out of the cell, the majority of investigators have postulated that the synthesis of polyglutamyl forms of MTX, as with natural folates, results in a retentive form of the drug [2,3]. Since MTX-polyglutamates appear to be formed to varying extents in different tissues, their lower efflux, compared to MTX, could result in a greater persistence of total drug in those tissues with a higher capacity for polyglutamate formation. Greater persistence of methotrexate at pharmacologically effective levels in responsive tumor cells versus normal proliferative tissue has, in fact, been associated [reviewed in Ref. 7] with the selective action of this agent in murine tumor models. Although these pharmacokinetic differences appear to be explained [7] to a large extent by differences in membrane transport, the role for polyglutamate formation has not been entirely excluded. In the present study, we have investigated the efflux of MTX-polyglutamates synthesized in L1210 cells following the administration of [^{3}H] MTX to leukemic mice. The extent of efflux of each derivative was determined by chromatographic analysis for [^{3}H] MTX and each [^{3}H] MTX-polyglutamate in both intracellular and extracellular compartments. A valid determination of extracellular accumulation of each [^{3}H] MTX-polyglutamate fraction could only be obtained in these experiments by effluxing in the presence of purified microbial dihydrofolate reductase added in excess to the cell suspension. Our results appear to show that efflux of MTX and each polyglutamate from these cells was the same.

Methods

C57BL/6 x DBA/2F$_1$ (BD2F$_1$) mice were transplanted with the ascitic form of the L1210 murine leukemia as described previously [8]. Four hours prior to removing leukemia cells, mice were injected s.c. with 48 mg/kg radioactive [^{3}H] MTX (Moravek Biochemicals, City of Industry, CA). L1210 cells were harvested from the peritoneal ascitic fluid in 0° buffered salts solution [9] with 10 mM dextrose. The cells were washed once and diluted in the same solution to a standard absorbance level (A_{600} = 3). Half the sample was set aside at 0° (T_0), and the remainder allowed to efflux at 37° for 20 min (T_{+20}). Both samples were then centrifuged at 0°, the supernatant fraction was set aside, and the cells were resuspended to 2 ml in 0° 0.14 M NaCl-0.01 M sodium phosphate, pH 7.4. An aliquot was assayed for cell number, and then both cells and the supernatant fractions were heated at 100° for 15 min to extract drug and inactivate cellular conjugase (carboxypeptidase) and polyglutamyl synthetase. The boiled samples were centrifuged to remove cellular debris. The cleared supernatant fractions were applied to 0.9 x 30 cm DEAE-Sephadex columns along with authentic MTX,

MTX+G_1 and MTX+G_2 (subscript denoting number of additional γ-glutamyl residues) markers. The chromatographic columns were developed with a linear gradient of 0.3 → 0.6 M NaCl in 0.05 M phosphate buffer, pH 7.0, collecting 2.0 ml fractions. Ultraviolet absorption of each fraction at 300 nm was measured to determine the elution positions of the authentic markers. The radioactivity of 1.0 ml of each fraction was assayed in 10 ml of Scintisol (Isolab, Akron, OH). A replicate series of experiments was performed with the addition of purified [10] Diplococcus pneumoniae dihydrofolate reductase in excess of drug binding equivalence to the salts solution used to resuspend the harvested cells prior to efflux. All other procedures remained the same as above.

Results and Discussion

The results of the experiment described above are given in Fig. 1. Chromatographic analysis of cell extract at time zero (upper left) revealed peaks of radioactivity corresponding to MTX, MTX+G_1, MTX+G_2 and MTX+G_3. Although authentic standards have been obtained only for MTX, MTX+G_1 and MTX+G_2, all peaks were (1) carboxypeptidase labile (except for MTX); (2) titration inhibitors of dihydrofolate reductase; and (3) eluted at equidistant ionic concentrations. A similar examination of the cells was carried out (upper right) after 20 min of incubation (T_{+20} cells). While the total intracellular drug level decreased by 57 percent, chromatographic analysis showed no change in the distribution of MTX and each of the metabolites shown (upper portion, Fig. 1). Since any retention of the conjugated forms would have markedly changed these profiles, this suggests that MTX and each of the polyglutamyl metabolites shown are leaving the cells at equal rates. The initial T_0 supernatant fraction (lower left) shows a trace amount of MTX following processing which is subtracted

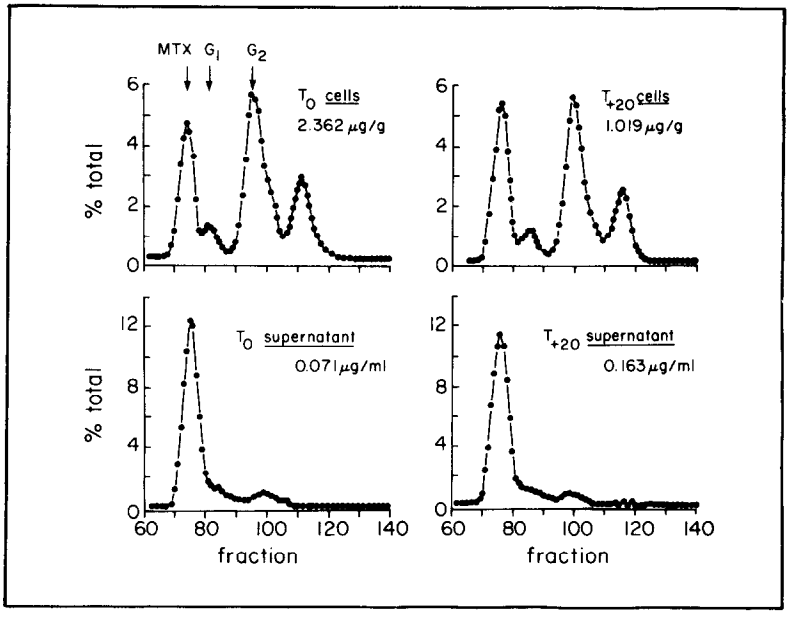

Fig. 1. Radioactive elution profiles after DEAE-Sephadex chromatography of heat-treated cellular or supernatant extracts. The vertical axis is percent total drug, which was 2.362 μg/g wet weight for T_0 cells, 1.019 μg/g for T_{+20} cells, after 20 min at 37°; 0.071 μg/ml for T_0 supernatant, 0.163 μg/ml for T_{+20} supernatant, after 20 min at 37°. The net intracellular decrease shown equals the net extracellular accumulation. Fractions (2.0 ml) of a 0.3 → 0.6 M linear NaCl gradient were collected. Elution positions of authentic markers MTX, MTX+G_1 and MTX+G_2 are indicated by arrows.

from the net T_{+20} supernatant profile. When the extracellular compartment of the cell suspension was examined after 20 min of incubation (lower right, Fig. 1), there was an accumulation of radioactivity equal to the intracellular decrease, but almost all (>95 percent) was in the form of MTX. In view of the result obtained for the cellular content after 20 min of incubation, the data relating to the extracellular accumulation of radioactivity could be explained in either one of two ways: (1) the polyglutamates effluxed as MTX following rapid intracellular hydrolysis, or (2) polyglutamyl forms of MTX effluxed intact, and were hydrolyzed after extracellular accumulation. Preliminary studies had demonstrated that MTX and MTX-polyglutamates bound to intracellular dihydrofolate reductase were not suitable substrates for either polyglutamyl synthetase or conjugase. Consequently, the same experiment was repeated and, in addition, a purified preparation of D. pneumoniae dihydrofolate reductase was added to the cell suspension. Our hope was that the extracellular microbial reductase might trap and preserve some intact polyglutamates as they effluxed. An analysis of the cellular contents before (T_0 cells) and after (T_{+20}) incubation gave the identical profiles, as shown in Fig. 2 (upper portion). Also, an analysis of the heat-treated T_{+20} supernatant fraction now showed the same distribution profile (lower right) as that obtained with the intracellular content before or after incubation. The total drug accumulating in the extracellular compartment was equal to the decrease in the intracellular fraction.

These results provide strong evidence for concluding that polyglutamyl derivatives of MTX efflux from L1210 cells as readily as MTX, itself. Since these metabolites are apparently not retentive forms of MTX in L1210 cells, polyglutamation is probably not relevant in determining drug persistence in this tissue. The necessity for adding dihydrofolate

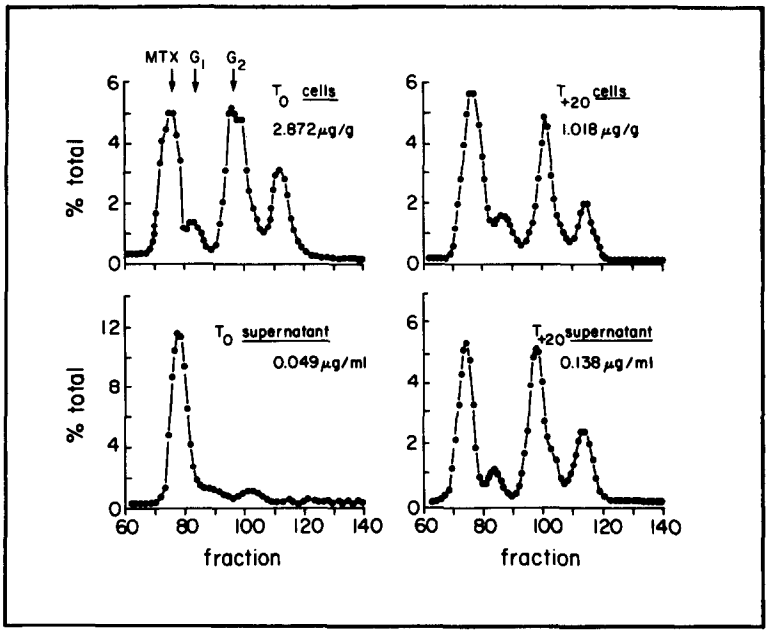

Fig. 2. Radioactive elution profiles after DEAE-Sephadex chromatography of heat-treated cellular or supernatant extracts, with D. pneumoniae dihydrofolate reductase added to the cell suspension. The vertical axis is total drug, which was 2.872 µg/g wet weight for T_0 cells, 1.018 µg/g for T_{+20} cells, after 20 min at 37°; 0.049 µg/ml for T_0 supernatant, 0.138 µg/ml for T_{+20} supernatant, after 20 min at 37°. The net intracellular decrease shown equals the net extracellular accumulation.

reductase to the cell suspension to demonstrate the extracellular accumulation of MTX-polyglutamates appears to relate to the presence [6] in the extracellular compartment of a hydrolytic enzyme(s). This enzyme activity could be derived from disrupted cells and(or) from ascitic fluid contamination. Recovery of extracellular polyglutamates does not occur when boiled microbial dihydrofolate reductase is employed. We assume that the native microbial dihydrofolate reductase added to the incubation medium tightly binds the methotrexate-polyglutamates, and prevents their enzymatic hydrolysis. Also, when [^{3}H] MTX was added to a sample of cell suspension with microbial enzyme, metabolism to polyglutamate forms did not occur.

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