Deoxyuridine Metabolism in Cultured Human Lymphoblasts Treated with Methotrexate

David J. Perez, Peter Slowiaczek, and Martin H. N. Tattersall

Ludwig Institute for Cancer Research (Sydney Branch), Blackburn Building, University of Sydney, Sydney, New South Wales, 2006, Australia

ABSTRACT

The deoxyuridine suppression test and labeled deoxyuridine incorporation studies assume a stable level of deoxyuridine phosphorylase (thymidine:orthophosphate deoxyribosyltransferase, EC 2.4.2.4) activity. We report a large increase in deoxyuridine phosphorylase activity in three of five cultured lymphoblast lines treated with $10^{-7}$ M methotrexate. In two of these lines, a parallel increase in deoxurydine incorporation into RNA was seen following methotrexate treatment. The high basal deoxyuridine phosphorylase activity in another lymphoblast line was associated with 80% incorporation of deoxurydine into RNA in untreated cells. Since methotrexate-induced changes in deoxyuridine phosphorylase activity were time dependent, the reliability of the deoxyuridine suppression test and labeled deoxyuridine incorporation into DNA as measures of thymidylate synthetase (EC 2.7.4.6) inhibition would also vary with time. Moreover, increases in deoxyuridine phosphorylase activity in methotrexate-treated cells may influence the metabolism of fluorouracil, a drug frequently used in combined treatment regimens.

INTRODUCTION

The metabolic basis of most techniques used to measure the activity of thymidylate synthetase is the anabolism of dUrd2 to dTMP. This assessment usually involves either the measurement of labeled dUrd incorporation into DNA (3, 16) or the measurement of labeled dThd incorporation in cells pretreated with high concentrations of dUrd (dUrd suppression test) (11, 17). The former technique assumes that inhibition of thymidylate synthetase or an increased dUMP level and reduction in the specific activity of labeled dUMP results in decreased incorporation of label into DNA. The same principle underlies the dUrd suppression test, in which synthesis of dTMP from exogenous dUrd is dependent on thymidylate synthetase action, and this, in turn, largely determines the specific activity of labeled dTMP formed when the cells are exposed to labeled dThd. In addition, increased dTTP levels allosterically inhibit thymidine kinase (EC 2.7.1.2), and this further reduces specific activity of labeled dTMP. Pelliniemi and Beck (20) have recently challenged these simplistic explanations of the dUrd suppression test by demonstrating that exogenous dUrd caused further effects, including partial reversal of the thymidylate synthetase blockade and competitive inhibition of dThd kinase, so that the incorporation of labeled dThd into DNA is not solely dependent on the dTMP pool size.

A corollary of these observations is that changes in dUrd/dThd catabolic activity will also influence the validity of the dUrd suppression test and labeled dUrd incorporation as measures of thymidylate synthetase activity. If dUMP accumulation resulting from thymidylate synthetase blockade increases dUrd catabolic activity, the usefulness of these tests would be in doubt.

We have measured dUrd phosphorylase in 5 cultured lymphoblast lines treated with $10^{-7}$ M MTX. Activity, was greatly increased in 3 cell lines, was marginally increased in 1 line, and remained unchanged in 1 line. Increased dUrd phosphorylase activity was associated with a parallel increase in [6-3H]dUrd incorporation into RNA in 2 of the cell lines studied.

MATERIALS AND METHODS

Cell Cultures. The human lymphoblast cell lines studied were: CCRF-CEM; RPMI-8402; JM (all T-lymphoblasts); and BALM (B-lymphoblast), obtained from Dr H. Lazarus, Sidney Farber Cancer Center, Boston, Mass., and Dr H. Zola, Flinders Medical Centre, Adelaide, Australia. The murine leukemic cell line L1210 was also investigated. All cell lines were grown in RPMI Medium 1640 supplemented with 10% fetal calf serum. Logarithmically growing control cells had the following doubling times: CCRF-CEM, 24 hr; RPMI-8402, 36 hr; JM, 24 hr; BALM, 36 hr; and L1210, 12 hr. Twenty-four hr prior to addition of $10^{-7}$ M MTX, logarithmically growing cells were suspended in fresh medium. All metabolic studies were performed at times related to the cell doubling of unperturbed logarithmically growing cells, namely, 0.25, 1, and 2 cell doubling times after addition of MTX.

[6-3H]dUrd Incorporation into DNA and RNA. At the appropriate assay times, duplicate samples of 2.5 x 10^6 cells in 2.5 ml of fresh medium (plus MTX, if appropriate) were incubated with 2.5 µCi of [6-3H]dUrd (2.5 µCi; final concentration, 5 x 10^{-6} M) (The Radiochemical Centre, Amersham, United Kingdom) at 37° for 20 min. The reaction was stopped by placing the tubes on ice for 10 min; then, the cells were washed with Hank’s balanced salt solution.

The separation of label incorporated into DNA and RNA fractions was based on the Schmidt-Thannhauser technique as modified by Munro and Fleck (19). Acid-soluble radioactivity was extracted by adding 1 ml of ice-cold 0.2 M PCA to the washed cell pellet followed by 15 min on ice to allow precipitation. After centrifugation for 10 min at 4000 rpm, the supernatant was decanted, and the precipitate was washed with a further 1 ml of 0.2% PCA.

Specific DNA and RNA fractionation was begun by incubating the acid-insoluble precipitate with 1 ml of 0.3 M NaOH at 37° for 1 hr to hydrolyze RNA. Following this, the reaction tubes were cooled at 4°, and the hydrolysate was reacidified to 0.2 M PCA by the addition of 3.0 M PCA. After a further 15 min at 4°, the hydrolysate and precipitate were separated by centrifugation, the precipitate was washed once with 0.2 M PCA, and the 2 supernatants were pooled to constitute the RNA fraction. To separate the remaining DNA fraction from protein and lipid, the precipitate was incubated with 0.5 M PCA at 70° for 30 min, resulting in DNA hydrolysis. The reaction tube was then cooled to 4° for 15 min, and the protein and lipid precipitate was separated by centrifugation. Following another 0.5 M PCA wash, the supernatants containing the DNA hydrolysate were pooled.

Aliquots of the DNA and RNA hydrolysates were placed in scintillation vials, and, after addition of scintillation fluid, the specific nuclear acid incorporation was measured and expressed as dpn/10^6 cells or dpm/μg of DNA or RNA (specific activity).

Quantitation of DNA and RNA. DNA was measured using a variant...
of the Burton diphenylamine reaction (10). These modifications enhanced the sensitivity of the assay and allowed its use below 10 μg of DNA per ml. The procedure involved addition of 2 ml of 4% diphenylamine in glacial acetic acid and 0.1 ml of aqueous acetaldehyde (1.6 mg/ml) to 2 ml of test solution of DNA in 0.1 M PCA. Following an overnight incubation at 30°, the absorbance at 595 nm was measured using a Varian Series 634 spectrophotometer.

A DNA standard curve was produced using calf thymus DNA in 0.1 M PCA, with a range of concentrations from 5 to 20 μg/ml.

RNA quantitation was performed by UV absorbance at 260 nm using RNA hydrolysate diluted to 0.1 M PCA. The standard curve was made using calf liver RNA in a concentration range of 5 to 20 μg/ml. Since protein absorbs UV light at 260 nm, hydrolyzed protein may represent a potential source of RNA quantitation error. To clarify this point, RNA hydrolysates from 6 experiments were subjected to a Lowry protein assay (18). In no instance was contaminating protein detected.

dUrd Phosphorylase Assay. This enzyme was assayed using the method described by Gallo and Perry (9). The reaction underlying the assay is the cleavage of dUrd (with 10⁻¹ M phosphate buffer) to uracil and deoxyribose 1-phosphate. Enzyme activity was expressed as nmol/hr/mg of protein and was linear with concentrations of cell-free protein extract ranging from 10 to 50 μg/ml of reaction mixture. Protein was estimated by the method of Lowry et al. (18).

RESULTS

The relative growth inhibitions produced in the 4 human lymphoblast lines by 10⁻⁷ M MTX are shown in Chart 1A, and the corresponding [6-³H]dUrd incorporations into DNA plus RNA (total nucleic acids)/10⁶ cells are shown in Chart 1B. [6-³H]-dUrd incorporation at 72 hr (2 times the doubling time for control cell cultures) was not possible with RPMI-8402 cultures because of inadequate cell numbers. Despite the similar growth-inhibitory effects produced by 10⁻⁷ M MTX on the 4 human cell lines, it can be seen that the patterns of [6-³H]-dUrd incorporation into total nucleic acids ranged from marked enhancement to almost complete suppression. L1210 cells behaved in precisely the same manner as did the CCRF-CEM cells in growth inhibition and dUrd incorporation (data not shown).

Chart 2 shows the results of fractionated [6-³H]dUrd incorporation expressed as DNA and RNA incorporation/10⁶ cells. Chart 2A shows that, in untreated CCRF-CEM cells, [6-³H]dUrd incorporation into DNA far exceeded incorporation into RNA. Treatment with MTX suppressed incorporation into both DNA and RNA. This result was similar in the murine L1210 cell line (data not shown). With RPMI-8402 cell (Chart 2B), there was significant incorporation of [6-³H]dUrd into RNA of untreated cells; however, DNA incorporation was still predominant. MTX markedly inhibited DNA incorporation, but RNA incorporation increased and, as a result, total nucleic acid incorporation persisted at a substantial level (40% of control). The very low levels of [6-³H]dUrd incorporation into the DNA of JM cells (Chart 2D) reflected the dThd kinase deficiency of this mutant cell line, and [6-³H]dUrd was incorporated predominantly into RNA of untreated cells. As with RPMI-8402 cells, MTX produced a dramatic increase in both total nucleic acids and RNA incorporation. Chart 2C shows that [6-³H]dUrd incorporation into RNA of untreated BALM cells exceeded DNA incorporation by a factor of 4 but, unlike JM cells, this cell line is not deficient in thymidine kinase. MTX produced marked inhibition of [6-³H]dUrd incorporation into DNA and moderate inhibition of RNA and total nucleic acids incorporation.

To ensure that the MTX-induced increases in [6-³H]dUrd RNA incorporation observed in RPMI-8402 and JM cells were not partially or completely due to a drug-induced increase in cellular
RNA content (unbalanced growth) (5), the label incorporation results were expressed as specific activities (Chart 3). Chart 3 also illustrates dUrd phosphorylase activity expressed as a percentage of control cultures, and the absolute values are shown in Table 1. The expression of label incorporation as specific activity did not diminish the MTX-induced enhancement of [6-3H]dUrd incorporation into RNA; in fact, the opposite effect occurred dUrd phosphorylase activities in the RPMI-8402 and JM (Chart 3, B and C) cells increased in parallel with the [6-3H] dUrd incorporation into RNA. A modest increase in BALM dUrd phosphorylase activity was seen following MTX, but this did not result in increased [6-3H]dUrd incorporation into RNA. dUrd phosphorylase activity was undetectable in untreated and treated CCRF-CEM cells (Chart 3A). Treatment of L1210 cells with 10^{-7} M MTX increased the dUrd phosphorylase activity in cell free extracts by 450% over 2 cell-doubling times. As with BALM cultures, however, no parallel increase in RNA incorporation of [6-3H]dUrd was observed.

DISCUSSION

Previous studies of labeled dUrd incorporation in neoplastic cells have shown a marked preponderance of incorporation into DNA compared to incorporation into RNA (7, 13), but the investigations reported here demonstrate significant [6-3H]dUrd incorporation into RNA in some lymphoblast cell lines. The pathways of dUrd metabolism are outlined in Chart 4. Significant incorporation into RNA was associated with either high basal or MTX-potentiated dUrd phosphorylase activity. The dThd kinase mutant line JM was an exception, because the basal [6-3H]dUrd incorporation into RNA was influenced by dThd kinase deficiency rather than by dUrd phosphorylase activity. The different patterns of [6-3H]dUrd incorporation into RNA were responsible for the poor correlation between lymphoblast growth inhibition caused by MTX and [6-3H]dUrd incorporation into total nucleic acids.

Although MTX treatment has been associated with induction of ribonucleotide reductase, dThd kinase, and thymidylate synthetase activities (4, 6), there are no previous reports of MTX-induced increases in dUrd phosphorylase activity. This potentiation was not seen with MTX concentrations of less than 10^{-7} M. Increased dUrd phosphorylase activity could result from enzyme activation, induction, or stabilization. Enzyme activation is an unlikely explanation, because the increase in activity was delayed, but further investigations will be necessary to identify the mechanism. The increase in dUrd phosphorylase activity has important implications for labeled dUrd incorporation studies and the dUrd suppression test. Increased dUrd phosphorylase activity will lower dUrd pools and may lower dUMP pools so contributing to the cytotoxic effect of MTX by reducing residual thymidylate synthetase activity maintained by elevated dUMP levels (20). In
addition, since dUrd phosphorylase may be an important 5-FUra anabolizing enzyme in some cell lines (21), the increased activity of this enzyme following MTX treatment may be relevant to the mechanism and schedule dependence of the MTX-5-FUra interaction (1, 2). This hypothesis is strengthened by the observation that the dUrd phosphorylase activity assayed in this study also accepted uridine and fluorouracil, thus leading to increased anabolism of 5-FUra to 5-fluourouridine and 5-fluorodeoxyuridine (data not shown).

Most previous studies using [3H]dUrd to study MTX-induced inhibition of dihydrofolate reductase or thymidylate synthetase (22) or MTX cytotoxicity (12, 14, 15) have not fractionated incorporation into DNA and RNA compartments. Some of these studies have reported poor correlations between in vitro [6-3H]dUrd incorporation and in vivo response to MTX (12, 15). It is possible that incorporation of [6-3H]dUrd into RNA may have contributed to these poor in vitro/in vivo correlations. The results reported here indicate that studies using [6-3H]dUrd for assessment of MTX-induced enzyme inhibition or cytotoxicity should include studies of incorporation into DNA and RNA. Even when [6-3H]dUrd incorporation into RNA in untreated cells is minimal, the fractionation step is still advisable because of possible enhancement of incorporation into RNA with MTX treatment.

In the presence of a thymidylate synthetase block, the dUMP pool size has an important bearing on the rate of residual dTMP production (20). This factor complicates the interpretation of the dUrd suppression test, because exogenous dUrd may artifactually increase thymidylate production and, in some cell lines, low dTTP pools dehinit dUrd deaminase (8), resulting in further dUMP production. The enhancement of dUrd phosphorylase activity by MTX in some cell lines may further complicate the use of the dUrd suppression test in MTX-treated cells by tending to reduce the dUMP pool. Since the dUrd phosphorylase activity enhancement was time dependent (Chart 9), the assessment of MTX-induced thymidylate synthetase blockade by the dUrd suppression test would also vary with time. Finally, enhanced dUrd phosphorylase activity would increase [3H]dTThd catabolism and thus decrease [3H]dTThd incorporation into DNA. This represents another non-dTMP-dTTP determinant of [3H]dTThd incorporation in the dUrd suppression test.

REFERENCES

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