Biochemical Basis for the Differential Sensitivity of Human T- and B-Lymphocyte Lines to 5-Fluorouracil

Anita A. Piper¹ and Richard M. Fox

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

ABSTRACT

The metabolism of 5-fluorouracil (FUra) was examined in two human lymphocyte cell lines, CCRF-CEM (T-leukemic) and LAZ-007 (Epstein-Barr virus-transformed B), which have widely differing FUra sensitivities. CEM cells have orotate phosphoribosyltransferase but no uridine or thymidine phosphorylase activity. Consistent with this, the CEM growth inhibition and the synthesis of fluorouridine triphosphate (FUTP) and fluorodeoxyuridine monophosphate (FdUMP) in CEM cells was substantially reduced by hypoxanthine and allopurinol. FUra growth inhibition of CEM cells was due to inhibition of both DNA and RNA synthesis, since thymidine only partially restored growth. Growth inhibition of LAZ cells, which possess all of the above FUra-activating enzymes, occurred at a lower FUra concentration than for CEM cells and was due (at FUra concentrations giving 95% growth inhibition) to inhibition of thymidylate synthesis, since thymidine completely restored growth. Consistent with this, the LAZ cells synthesize FdUMP at much higher rates than do CEM cells. FdUMP synthesis was not significantly inhibited by hypoxanthine but was activated by deoxyinosine and strongly inhibited by thymidine, leading us to conclude that LAZ cells synthesize FdUMP predominantly via thymidine phosphorylase. At higher (>10⁻⁶ M) FUra concentrations, thymidine only partially restored LAZ cell growth, indicating impairment of RNA metabolism apparently due to FUTP synthesized via orotate phosphoribosyltransferase since (a) substantial protection was obtained by addition of hypoxanthine to FUra plus thymidine and (b) FUTP synthesis in LAZ cells was strongly inhibited by hypoxanthine. Thus, the increased LAZ sensitivity of LAZ cells is due primarily to their greater rate of FdUMP synthesis via thymidine phosphorylase. Impairment of RNA metabolism occurred in both cell lines at similar FUra concentrations and was due to FUTP synthesis via orotate phosphoribosyltransferase, the activity of which is similar in LAZ and CEM cells.

INTRODUCTION

The antimetabolite FUra² is widely used in the treatment of cancer of the breast (5) and gastrointestinal tract (30), but responses are obtained in only 10 to 30% of patients with advanced disease. FUra is activated in vivo by one or more of 3 alternative routes (Chart 1). It may be converted to FUMP either directly by orotate phosphoribosyltransferase (Pathway I) or by the sequential action of uridine phosphorylase and uridine kinase (Pathway II). FUMP may then be converted to FUTP which, after incorporation into RNA, can interfere with the synthesis and function of all classes of RNA (17, 22, 47, 48). Studies with both tissue culture systems (27, 44, 46) and with animals (20, 38, 41) indicate that in certain circumstances such interference with RNA metabolism contributes to cytotoxicity by FUra. Alternatively, FUMP may be converted (after reduction of FUDP by ribonucleotide reductase (23)) to FdUMP, which is an inhibitor of thymidylate synthetase (19) and hence DNA synthesis. Thus, activation of FUra by Pathways I or II may give rise to inhibition of DNA and/or RNA metabolism. The third route of FUra activation (that route catalyzed by thymidine phosphorylase and thymidine kinase) results in the synthesis of FdUMP only (Chart 1), therefore resulting in inhibition of DNA synthesis only.

The sensitivity to FUra and the mode of cytotoxicity induced by FUra in different tissues presumably reflects differences in their metabolism of FUra to the potentially cytotoxic products FdUMP and FUTP. However, the problems involved in studying human tissues make it extremely difficult to determine the relative importance of the 3 pathways of FUra activation in normal and malignant tissues. One approach to this problem is to interpret in vitro biochemical parameters such as enzyme determinations on tissue samples within the framework of model cell culture systems. We report here the results of an investigation into the pathways for FUra metabolism in the 2 human lymphocyte cell lines, CCRF-CEM (T-acute lymphoblastic leukemic) and LAZ-007 (Epstein-Barr virus-transformed B-cells). These cell lines serve as useful models inasmuch as they have major differences in their anabolism of FUra which correlate with their sensitivity to growth inhibition by FUra and which can be exploited to modulate their sensitivity to FUra. Portions of this work have been presented in preliminary form elsewhere (35).

MATERIALS AND METHODS

Cell Culture and Growth Inhibition Studies. The Epstein-Barr virus-transformed B-lymphocyte cell line LAZ-007 (kindly provided by Dr. H. Lazarus, Sidney Farber Cancer Institute, Boston, Mass.) and the acute lymphoblastic leukemic T-lymphocyte cell line CCRF-CEM (29) were maintained in suspension culture in exponential growth in Roswell Park Memorial Institute Medium 1640 containing 10% fetal calf serum. Cultures were checked periodically for Mycoplasma contamination (28, 39). Cells at an initial cell density of 1 x 10⁵ cells/ml were exposed to varying concentrations of FUra plus other drugs (added simultaneously with FUra), as indicated in the text. At 72 hr, the change in cell density (trypan blue-excluding cells) of drug-treated cells was expressed as a percentage of that in control cells. Cells used for biochemical studies were grown in exponential phase to approximately 10⁶ cells/ml.

Conversion of [2-¹⁴C]Uridine to Deoxynucleotides. The effect of hydroxyurea on ribonucleotide reduction was determined by preincu-
bating (30 min, 37°C) cells (2 × 10^6 cells/ml, 3 ml) with 1 mM hydroxyurea and then adding 0.5 μCi [2-14C]uridine (57 mCi/mmols). After a further 60-min incubation, the cells were centrifuged (200 × g for 5 min) and extracted with 1 ml 0.3 N PCA (4°C), and the precipitate was washed once with 1 ml 0.3 N PCA (4°C). The 2 PCA supernatants were combined, neutralized with K2CO3, and freeze-dried after centrifugation to remove insoluble KIO4. The freeze-dried samples were incubated (2 hr, 37°C) in 0.3 ml 50 mM Tris-HCl (pH 9.0)-4 mM MgCl2-0.5 mM UMP, containing snake venom (3 mg/ml; Crotalus adamanteus; Sigma Chemical Co., St. Louis, Mo.) to convert nucleotides to nucleosides (6). After addition of 25 μl 3 N PCA and removal of protein by centrifugation (10,000 × g for 5 min), the samples were neutralized, and uridine, dUrd, and thymidine were separated by elution from a C18-pBondapak column (Waters Associates, Inc., Milford, Mass.) using a linear gradient over 5 min of from 0 to 15% (v/v) methanol-H2O (2 ml/min). Two-ml fractions were counted for radioactivity in 10-ml Aquasol scintillation fluid.

The DNA-containing PCA precipitate was dissolved in 1 ml 0.3 N NaOH (90 min, 37°C) to hydrolyze RNA, and then DNA was precipitated with 0.22 ml 3 N PCA (4°C) and collected by filtration onto Whatman GFA discs. After 2 PCA washes, the discs were placed in 0.5 ml Protosol (50°C, 30 min); then, 20 μl glacial acetic acid plus 5 ml Aquasol were added, and radioactivity was counted. The total radioactivity in free dUrd and thymidine nucleotides plus that incorporated into DNA was determined and expressed as a percentage of that obtained in the absence of hydroxyurea.

**Conversion of [6-14C]Orurate to Nucleotides.** Cells (approximately 10^6 cells/ml; 10 ml) were preincubated (60 min, 37°C) with 1 mM allopurinol or hydroxanthine; then, 0.5 μCi [6-14C]orurate (6.1 mCi/mmol) was added, and after 3 hr of incubation the cells were centrifuged, extracted, and washed once with PCA. The 2 PCA supernatants were combined, neutralized, centrifuged to remove KIO4, and freeze-dried in preparation for analysis of nucleotides by HPLC (see below). The RNA- plus DNA-containing PCA precipitate was hydrolyzed in 0.5 ml Protosol and counted for radioactivity as described above. The total radioactivity in each nucleotide was much lower than that in DNA (except for hydroxyurea cultures) and moreover followed the same trend as did the label in DNA, we subsequently measured radioactivity in DNA only for all except hydroxyurea cultures. The inhibition of dUrd conversion by FUra was expressed as a percentage of the conversion obtained in the absence of FUra but in the presence of the modulating agent (allopurinol, hydroxanthine, or hydroxyurea) where applicable.

**Measurement of Nucleotide Synthesis from [6-3H]FUra.** Ten-ml cell suspensions (approximately 10^6 cells/ml) were preincubated with 1 mM hydroxyurea (30 min) or 1 mM allopurinol or hydroxanthine (60 min). [6-3H]FUra (18 Ci/mmols; 0.05 μCi for LAZ cells; 0.2 μCi for CEM cells) plus unlabeled FUra as required to produce the final concentrations indicated in “Results” was then added, and after a 3-h incubation the cells were spun down, extracted, and washed once with PCA. The 2 PCA supernatants were combined, neutralized, and freeze-dried in preparation for measurement of FdUMP, FUMP, UDP, and FUTP by HPLC (see below). RNA in the PCA precipitate was hydrolyzed in 2 ml 0.3 N NaOH (90 min, 37°C) and, after precipitation of DNA by addition of PCA to 0.3 N final concentration, the absorbance at 260 nm and the radioactivity of the RNA-containing PCA supernatant were measured. The amounts of each FUra-nucleotide species and of FUra incorporated into RNA are expressed as pmol/μg DNA unit of RNA.

**HPLC Analysis of Nucleotides.** Freeze-dried PCA-soluble cell extracts were resuspended in 0.3 ml H2O and, after adjustment of pH to between 5 and 7, were centrifuged to remove precipitated salts. Nucleotides were eluted from a Brownlee RP18 (10 μm) column (Brownlee Labs, Inc., Santa Clara, Calif.) using 5 mM tetrabutylammonium phosphate buffer, pH 7.0 (2 ml/min, 25 min), followed by a linear gradient of from 0 to 60% (v/v) methanol-5 mM tetrabutylammonium phosphate over 20 min (2 ml/min). Unlabeled CMP, UMP, OMP, UDP, UTP, and FdUMP were chromatographed as markers (A254 nm), with radiolabeled FdUMP, FUMP, UDP, and UTP being identified by their position relative to these markers. Two-ml fractions were collected and counted for radioactivity throughout the elution program. HPLC analysis was performed on a Waters Associates, Inc., system.

**Enzyme Assays.** Cells were resuspended (approximately 3 × 10^8 cells/ml) in 50 mM Tris-HCl, pH 8.4 (4°C), and disrupted by sonication (4 times for 10 sec, with 30-sec rest intervals; 4°C). Enzyme assays were performed in duplicate at 3 different enzyme concentrations on the supernatant obtained after centrifugation of the homogenate at 100,000 × g for 1 hr. The assays were incubated at 37°C for 30 min and stopped by placing in a boiling-water bath for 2 min. Orotate and FUra phosphoribosyltransferases were assayed in reaction mixtures (100 μl) containing 50 mM Tris-HCl, pH 7.5 (37°C); 2.5 mM MgCl2, 2.5 mM phosphoribosylpyrophosphate; 1.0 mM dihydrothorretol; 0.2 mM [6-14C]orotate (10 μCi/mmol) or 1.0 mM [6-3H]FUra (2 ml/mmol); and cell supernatant. Lactose and base were separated as described by Reyes and Guganig (36) and counted for radioactivity in OCS (organic counting scintillant). Thymidine and FdUrd phosphorylase were assayed in reaction mixtures (100 μl) containing 50 mM Tris-HCl, pH 7.5 (37°C), 50 mM sodium succinate, pH 7.2, 10 mM deoxyribosyl 1-phosphate, 1 mM [2-14C]thymine (2 mM/mmol) or 1 mM [6-14C]FUra (2 mM/mmol), and cell supernatant. Lactose and base were separated as described by Gallo et al. (16) except that chromatography was performed on silica gel thin-layer sheets instead of paper) and counted for radioactivity in OCS (organic counting scintillant). Thymidine and FdUrd phosphorylase were assayed as described above for thymidine phosphorylase except for the substitution of 10 mM ribose 1-phosphate for deoxyribosyl 1-phosphate and of 1 mM [2-14C]aracil (2 mM/mmol) for [2-14C]thymine. Protein was assayed by the method of Lowry et al. (26) with bovine albumin as standard.


---


**Formula:**

\[ 	ext{FUra} \xrightarrow{\text{PRPP}} \text{PRPP} \]
Metabolism of FUra in Human T- and B-Cell Lines

**Chemicals.** All radiochemicals and liquid scintillation cocktails were obtained from the Radiochemical Centre, Amersham, England, except [6-3H]FUra which was obtained from Moravek Biochemicals, Inc., Brea, Calif., and Protosol, which was obtained from New England Nuclear, Boston, Mass. Cell culture media and fetal calf serum were from Grand Island Biological Co., Grand Island, N. Y. FUrd and FdUrd were obtained from Calbiochem-Behring Corp., San Diego, Calif. Tetrabutylammonium phosphate was obtained from Waters Associates, Inc. All other biochemicals were from Sigma.

**RESULTS**

**Inhibition of LAZ and CEM Cells by FUra, FUrd, FdUrd.** The LAZ cells show a log greater sensitivity to FUra than do CEM cells (Table 1). However, they are slightly less sensitive than CEM cells to the deoxynucleoside FdUrd and have similar sensitivity to the nucleoside FUrd (Table 1), indicating that the differential sensitivity of the LAZ and CEM cells to FUra reflects differences in their initial conversion of FUra to either FUMP, FUrd, or FdUrd by orotate phosphoribosyltransferase (Pathway I), uridine phosphorylase (Pathway II), or thymidine phosphorylase (Pathway III), respectively (Chart 1).

**Activities of FUra-activating Enzymes.** The CEM cells have no detectable thymidine or uridine phosphorylase activity either with the natural substrate or with FUra (Table 2). However, they do have phosphoribosyltransferase activity measured both with orotic acid and with FUra, indicating that CEM cells metabolize FUra via Pathway I. The LAZ cells have phosphoribosyltransferase activity similar to that of the CEM cells but in addition have uridine and thymidine phosphorylase activities (Table 2) and thus are potentially capable of activating FUra by all 3 pathways.

**Modulation of FUra Sensitivity in CEM and LAZ Cells.** Hypoxanthine (42, 49) and allopurinol (40) have been demonstrated previously to protect several cell lines from growth inhibition by FUra, most likely by reducing phosphoribosyl pyrophosphate levels (10, 42) and therefore orotate phosphoribosyltransferase activity (9). In addition, OMP decarboxylase is inhibited by metabolites of allopurinol (3, 15), resulting in elevation of orotate (1, 13, 40), which is a competitive inhibitor of FUra activation by orotate phosphoribosyltransferase (37). As shown in Table 3, hypoxanthine (3 × 10^{-4} M) gives considerable protection to CEM cells from growth inhibition by FUra, increasing the FUra ID_{so} concentration some 12-fold (Table 3; Chart 2). Allopurinol (10^{-4} M) also reduced the growth inhibition by FUra of CEM cells, but the protection was limited by the growth inhibition by allopurinol itself (ID_{so} approximately 3 × 10^{-4} M). Addition of thymidine to circumvent the block of thymidylate synthetase reduced the growth inhibition of CEM cells by FUra (Table 3) but never completely restored growth (Chart 2), indicating that the growth inhibition is not due solely to inhibition of thymidylate synthesis.

In contrast, the growth inhibition of LAZ cells by FUra concentrations of up to 10^{-6} M (which results in almost complete growth inhibition) was completely abolished by thymidine (Chart 2) but was not reduced by either hypoxanthine or allopurinol (Table 3). However, at FUra concentrations greater than 10^{-6} M, thymidine did not completely restore growth, and the growth inhibition remaining in the presence of thymidine was substantially reduced by hypoxanthine (Chart 2; Table 3). Together these results suggest that at FUra less than 10^{-6} M growth inhibition is due to inhibition of thymidylate synthesis by FdUMP synthesized via Pathway III and/or Pathway II but that at higher FUra concentrations inhibition of other cellular processes (presumably RNA metabolism), mediated by FUra-ribonucleotides synthesized predominantly via Pathway I, also contribute to cytotoxicity.

While we cannot distinguish between the contributions of Pathways II and III to FdUMP synthesis, the observation that deoxynosine strongly potentiates in a dose-dependent manner the growth inhibition by FUra of LAZ cells suggests that Pathway III (thymidine phosphorylase) can be a route for FdUMP synthesis in these cells (Table 3). Deoxynosine is converted to hypoxanthine plus deoxyribose 1-phosphate by purine nucleoside phosphorylase (33) and, since hypoxanthine itself has no effect on FUra growth inhibition of LAZ cells, it is likely that the potentiation is due to the increase in deoxyribose 1-phosphate concentration. Deoxynosine did not potentiate FUra toxicity in

**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>FUra (µM)</th>
<th>FUrd (nm)</th>
<th>FdUrd (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCRF-CEM</td>
<td>5.6 ± 1.4</td>
<td>22 ± 3 (3)</td>
<td>6.0 ± 0.8 (4)</td>
</tr>
<tr>
<td>LAZ-007</td>
<td>0.44 ± 0.17</td>
<td>20 ± 5 (3)</td>
<td>16 ± 5 (3)</td>
</tr>
</tbody>
</table>

* a Drug exposure was for 72 hr.
* b Mean ± S.E. of separate determinations.
* c Numbers in parentheses, number of experiments.

**Table 2**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Activity (nmol/hr/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orotate</td>
<td>Orotic acid</td>
<td>52 ± 12 a</td>
</tr>
<tr>
<td>phosphoribosyltransferase</td>
<td>FUra</td>
<td>75 ± 16</td>
</tr>
<tr>
<td>Uridine phosphorylase</td>
<td>Uracil</td>
<td>&lt;3 b</td>
</tr>
<tr>
<td></td>
<td>FUra</td>
<td>139 ± 30</td>
</tr>
<tr>
<td>Thymidine</td>
<td>Thymine</td>
<td>&lt;3 b</td>
</tr>
<tr>
<td>phosphoribosyltransferase</td>
<td>FUra</td>
<td>&lt;3 b</td>
</tr>
</tbody>
</table>

* a Mean ± S.E. of 3 separate experiments.
* b The limit of detection in the phosphorylase assays was 3 nmol/hr/mg protein.

**Table 3**

<table>
<thead>
<tr>
<th>Modulator</th>
<th>Concentration (m)</th>
<th>ID_{so} for FUra (µA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>5.6 ± 1.4 (8) b</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>3 × 10^{-4}</td>
<td>68 ± 22 (3)</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>1 × 10^{-4}</td>
<td>20 ± 5 (1)</td>
</tr>
<tr>
<td>Thymidine</td>
<td>5 × 10^{-5}</td>
<td>20 ± 5 (3)</td>
</tr>
<tr>
<td>Thymidine + hypoxanthine</td>
<td>5 × 10^{-5}</td>
<td>69 ± 11 (2)</td>
</tr>
<tr>
<td>Deoxynosine</td>
<td>1 × 10^{-4}</td>
<td>11 (1)</td>
</tr>
<tr>
<td></td>
<td>1 × 10^{-3}</td>
<td>0.114 ± 0.02 (2)</td>
</tr>
</tbody>
</table>

* a Mean ± S.E. of separate determinations.
* b Numbers in parentheses, number of experiments.
* c The limit of detection was 3 nmol/hr/mg protein.

SEPTEMBER 1982

3755
droxyurea may result in elevation of endogenous substrate or hypoxanthine and of uridine nucleotide reduction by hydantoin and thus should effectively inhibit FdUMP synthesis via either deoxynucleotide production from [14C]uridine in CEM and LAZ pools which may, by dilution of radioactive substrate, result in completely abolished deoxynucleotide production in both cell lines (Table 4). Table 4 also shows the effect of hydroxyurea on conversion of [14C]orotate to nucleotides in CEM cells, reducing nucleotide formation to only 56% of control. Hypoxanthine was also a good inhibitor of orotate conversion to 8 and 11% of control, respectively. As shown in Table 4, both allopurinol and hypoxanthine reduced [14C]orotate to nucleotides in CEM cells by 95% (Table 5; Chart 3), but this inhibition was abolished by hydroxyurea and substantially reduced by allopurinol and hypoxanthine (Table 5). Since the inhibition of dUrd conversion is not linearly proportional to concentration at high FUra concentrations (Table 5; Chart 3), it is difficult to determine the extent of reduction of FdUMP synthesis produced by allopurinol and hypoxanthine. However, the residual inhibition produced by 5 × 10⁻⁴ M FUra in the presence of these agents was less than that at 5 × 10⁻⁵ M FUra suggesting, if FdUMP synthesis is linear with concentration at 5 × 10⁻⁴ M FUra, at least a 10-fold reduction in FdUMP synthesis. Direct measurement of FUra-nucleotide synthesis at 5 × 10⁻⁵ M and 5 × 10⁻⁴ M FUra (also shown in Table 5) indicates that FdUMP synthesis was essentially still linear with FUra concentration at 5 × 10⁻⁴ M FUra; thus, the nonlinearity of dUrd conversion is presumably due to saturation of thymydylate synthetase rather than to saturation of the pathway for synthesis of dUMP. Hydroxyurea, allopurinol, and hypoxanthine all reduced FUra levels to less than 21% of control levels, and allopurinol and hypoxanthine reduced total FUra-ribonucleotide synthesis approximately 10-fold (Table 5). Thus, the results obtained by direct measurement of FUra-nucleotide levels were similar to those obtained from the dUrd conversion experiments and confirmed the prediction from enzyme measurements that CEM cells activate FUra via orotate phosphoribosyltransferase (Pathway I).

Effect of Modulators on FUra Metabolism in CEM Cells. Consistent with the greater sensitivity of LAZ cells to growth inhibition by FUra, much lower FUra concentrations were required to inhibit dUrd conversion to an extent similar to that in CEM cells (Chart 3). The LAZ cells accumulated free FdUMP at a much faster rate than did CEM cells, having a similar level of FdUMP at 5 × 10⁻⁴ M FUra [approximately 2.5 pmol/A₂₆₀ RNA (Table 6)] as did CEM cells at a 10-fold higher (5 × 10⁻⁵ M) concentration [approximately 1.4 pmol/A₂₆₀ RNA (Table 5)]. A concentration of 5 × 10⁻⁶ M FUra inhibited dUrd conversion in LAZ cells by 95%, but in contrast to CEM cells neither hypoxanthine nor allopurinol had any significant effect on this inhibition (Table 6). Consistent with this, hypoxanthine and allopurinol reduced FdUMP levels only slightly (Table 6), overestimation of inhibition. However, it is nonetheless clear from these results that all of these agents, with the possible exception of allopurinol on LAZ cells, will inhibit substantially the activity of the relevant enzymes and thus of FUra metabolism via these enzymes.

Effect of Modulators on FUra Metabolism in LAZ Cells. Prior to biochemical study of FUra metabolism, the effectiveness of allopurinol and hypoxanthine as inhibitors of Pathway I (orotate phosphoribosyltransferase) was tested using the natural substrate orotate, which is converted to nucleotides solely by this pathway. As shown in Table 4, both allopurinol and hypoxanthine (1 mM each) were excellent inhibitors of conversion of [¹⁴C]orotate to nucleotides in CEM cells, reducing nucleotide formation to 8 and 11% of control, respectively. Hypoxanthine was also a good inhibitor of orotate conversion to nucleotides in LAZ cells, but allopurinol was a relatively poor inhibitor, reducing nucleotide formation to only 56% of control (Table 4). Table 4 also shows the effect of hydroxyurea on deoxynucleotide production from [¹⁴C]uridine in CEM and LAZ cells. At a concentration of 1 mM, hydroxyurea almost completely abolished deoxynucleotide production in both cell lines and thus should effectively inhibit FdUMP synthesis via either Pathway I or II.

We note that inhibition of orotate conversion by allopurinol or hypoxanthine and of uridine nucleotide reduction by hydroxyurea may result in elevation of endogenous substrate pools which may, by dilution of radioactive substrate, result in CEM cells, which have no thymidine phosphorylase activity but rather gave slight protection, presumably due to the rescue effect of the hypoxanthine formed from deoxyinosine (Table 3).

Effect of Modulators on Orotate and Uridine Metabolism. Prior to biochemical study of FUra metabolism, the effectiveness of allopurinol and hypoxanthine as inhibitors of Pathway I (orotate phosphoribosyltransferase) was tested using the natural substrate orotate, which is converted to nucleotides solely by this pathway. As shown in Table 4, both allopurinol and hypoxanthine (1 mM each) were excellent inhibitors of conversion of [¹⁴C]orotate to nucleotides in CEM cells, reducing nucleotide formation to 8 and 11% of control, respectively. Hypoxanthine was also a good inhibitor of orotate conversion to nucleotides in LAZ cells, but allopurinol was a relatively poor inhibitor, reducing nucleotide formation to only 56% of control (Table 4). Table 4 also shows the effect of hydroxyurea on deoxynucleotide production from [¹⁴C]uridine in CEM and LAZ cells. At a concentration of 1 mM, hydroxyurea almost completely abolished deoxynucleotide production in both cell lines and thus should effectively inhibit FdUMP synthesis via either Pathway I or II.

We note that inhibition of orotate conversion by allopurinol or hypoxanthine and of uridine nucleotide reduction by hydroxyurea may result in elevation of endogenous substrate pools which may, by dilution of radioactive substrate, result in

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>% of conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CEM</td>
</tr>
<tr>
<td>Orotate</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Allopurinol</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Hypoxanthine</td>
<td>11</td>
</tr>
<tr>
<td>Uridine</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Hydroxyurea</td>
<td>2</td>
</tr>
</tbody>
</table>

Product formation (nucleotides from orotate and deoxynucleotides from uridine) in the presence of inhibitors (1 mM allopurinol or hypoxanthine for orotate and 1 mM hydroxyurea for uridine) is expressed as a percentage of that obtained in the absence of inhibitor.
suggesting that the residual (approximately 20%) hydroxyurea-fold higher hydroxyurea concentration (results not shown), was reduced to an extent (Table 6) similar to their inhibition of nucleotide synthesis from orotate (Table 4), indicating that Pathway I is not the major route of FdUMP synthesis in LAZ cells. The rate of FUTP synthesis was similar to that of CEM cells (Table 7), a finding consistent with their similar orotate phosphoribosyltransferase activities (Table 2).

The inhibition of dUrd conversion by FUrA was reduced by hydroxyurea, but only slightly, from 95 to 85% (Table 6). However, the nonlinearity of inhibition at 5 x 10^-5 M FUrA is such (Chart 3) that this apparently small reduction of inhibition will, if FdUMP synthesis is linear with FUrA concentration, correspond to a substantial reduction in FdUMP levels. This was confirmed by the finding that hydroxyurea reduced the synthesis of FdUMP by approximately 80% (Table 6), suggesting that Pathway II is the major route of FdUMP synthesis in LAZ cells.

In view of the fact that Pathways I and II converge after FUMP synthesis before the branch point between reduction or further phosphorylation, we thought it surprising that FdUMP should be synthesized predominantly via Pathway II, when FUTP was synthesized predominantly via Pathway I. Thus, we measured FdUMP synthesis in the presence of deoxyinosine plus hydroxyurea and found that hydroxyurea substantially inhibited (approximately 71%) the deoxyinosine-driven FdUMP synthesis in LAZ cells but left FUrA-ribonucleotide levels unchanged (Table 7). These results indicate that hydroxyurea can inhibit (possibly indirectly) FdUMP synthesis via Pathway III, and therefore the above conclusion, based on the hydroxyurea sensitivity of FdUMP synthesis, that pathway II is the major route of FdUMP synthesis in LAZ cells is not valid.

The more likely possibility, that LAZ cells synthesize FdUMP predominantly via thymidine phosphorylase (Pathway III), was supported by the observation that thymidine (at concentration equivalent to that of FUrA) strongly inhibited (approximately 90%) FdUMP synthesis in LAZ cells (Table 7). Although phosphorylation of thymidine may elevate deoxyribose 1-phosphate levels, thereby increasing FdUrd synthesis, thymidine phosphorylase is believed to function predominantly in the catabolic direction in vivo (4); thus, in short-term experiments, the steady-state levels of FdUrd are likely to be much lower (at equivalent exogenous FUrA and thymidine concentrations) than those of thymidine. Thymidine will therefore competitively inhibit the conversion of FdUrd to FdUMP by thymidine kinase. Consistent with the proposed mechanism of inhibition of FdUMP synthesis in LAZ cells, thymidine did not inhibit FdUMP synthesis in CEM cells or FUrA-ribonucleotide synthesis in either cell line (Table 7).

Inhibitions of dUrd conversion are expressed as percentages of the conversion in the absence of FUrA but in the presence of the appropriate modulating agent.

**Table 5**

| FUrA (mM) | Modulator | % of inhibition of dUrd conversion | Relative nucleotide synthesis
|-----------|-----------|-----------------------------------|-----------------------
| 5 x 10^-5 | None      | 71 ± 9 (<15 ± 4) (1.3 ± 0.4)     | FdUMP 10 ± 2 (166 ± 37) |
| 5 x 10^-4 | None      | 100 ± 10(9.3 ± 2.3)               | FUrA-ribonucleotide    |
|           | Hydroxyurea | 21 ± 14 (19 ± 10)                |                       |
|           | Allopurinol | 10 ± 2 (10 ± 2)                  |                       |
|           | Hypoxanthine | 52 ± 9 (52 ± 9)                  |                       |

* a Inhibitions of dUrd conversion are expressed as percentages of the conversion in the absence of FUrA but in the presence of the appropriate modulating agent.
* b Amounts of FdUMP and of total (both free and incorporated into RNA) FUrA-ribonucleotides synthesized in each case are expressed as a percentage of that produced at 5 x 10^-4 M FUrA. The amount of FUrA incorporated into RNA represented approximately 40% of the total FUrA-ribonucleotides synthesized. The reduction, caused by allopurinol or hypoxanthine to FUrA incorporated into RNA, was similar to that shown for the total FUrA-ribonucleotides.
* c Mean ± S.E. of 4 separate experiments.
* d Numbers in parentheses, absolute amounts (pmol/A260 nm RNA) of FdUMP and of FUrA-ribonucleotides synthesized at 5 x 10^-5 M and at 5 x 10^-4 M FUrA. Mean ± S.E. of 3 separate experiments.
* e Amounts of FdUMP synthesized by CEM cells are so low compared to the amounts of ribonucleotides formed and to the levels of radioactive contaminants present in the [3H]FUrA that where FdUMP formation was inhibited it was not possible to measure the levels more accurately than as shown.

**Figure 1**

Chart 3. Comparison between FUrA concentrations required to inhibit growth (72-hr exposure to FUrA; O, LAZ; C, CEM) and dUrd conversion (O, LAZ; C, CEM; measured as described in "Materials and Methods").
Consistent with the finding that CEM cells have orotate phosphoribosyltransferase but no uridine or thymidine phosphoribosylase activity, the synthesis of both FdUMP and FUTP in CEM cells was strongly inhibited by allopurinol and by hypoxanthine. Similarly, the growth inhibition of CEM cells by FURA (which was only partially reduced by thymidine and thus was due to inhibition of both DNA and RNA metabolism) was markedly reduced by hypoxanthine.

The synthesis of FUTP in LAZ cells, which have all 3 FURA-activating enzymes, was also strongly inhibited by hypoxanthine, but the synthesis of FdUMP was little affected, indicating that these cells synthesize FUTP predominantly via Pathway I but synthesize FdUMP mainly via Pathway II. Although the rate of FUTP synthesis was similar in CEM and LAZ cells, the rate of FdUMP synthesis was much greater in LAZ than in CEM cells. Consistent with this, the greater sensitivity to growth inhibition by FURA (3x10⁻⁶ M) of LAZ compared to CEM cells was due predominantly to inhibition of thymidylate synthesis and was not reduced by hypoxanthine.

The markedly potentiation of FdUMP synthesis and of FURA metabolism in LAZ cells indicates that these cells synthesize FUTP predominantly via Pathway II and/or Pathway III (which was only partially reduced by thymidine and thus was due to inhibition of both DNA and RNA metabolism) was markedly reduced by hypoxanthine.

The marked potentiation of FdUMP synthesis and of FURA metabolism in LAZ cells suggests that the major route of FdUMP synthesis in these cells was Pathway III, in which thymidine inhibited FdUMP synthesis by competitively inhibiting the phosphorylation of FdUrd to FdUMP. The level of thymidine used (50 μM) was well below the growth-inhibitory concentrations for LAZ cells [ID₅₀, 2 mM (12, 14)], making it unlikely that the inhibition of FdUMP synthesis was due to inhibition of ribonucleotide reductase by elevated dTTP pools. Even in CEM cells, where inhibition of ribonucleotide reductase results in growth inhibition at much lower thymidine levels [ID₅₀, 30 μM (12, 14)], FdUMP levels were not reduced by a 3-hr exposure to 50 μM thymidine. We conclude, therefore, that LAZ cells synthesize FdUMP predominantly via Pathway III and at much faster rates than its synthesis in CEM cells via Pathway I. High thymidine phosphoribosyltransferase (Pathway III) activity appears to be a characteristic feature of human B-lymphocytes (11), and this may account for their consistently greater sensitivity to FURA compared to T-cells (32). At FURA concentrations >10⁻⁶ M, thymidine only partially restored LAZ cell growth, but the inhibition remaining in the presence of thymidine was substantially reduced by hypoxanthine, indicating that it resulted from impairment of RNA metabolism by FUTP synthesized via Pathway I. Consistent with the similar rates of FUTP synthesis in CEM and LAZ, the non-thymidine-reversible inhibition of LAZ cell growth occurred at FURA concentrations closer to those which inhibited CEM cell growth.

Since phosphoribosyl pyrophosphate, ribose 1-phosphate,
and deoxyribose 1-phosphate may all be rate limiting for their respective enzymes, modulation of their concentration may alter FUra sensitivity in vivo. Thus, purines which lower phosphoribosyl pyrophosphate levels (10, 42) decrease FUra sensitivity in these and other cell lines (40, 42, 49). Conversely, methotrexate pretreatment has been shown to elevate phosphoribosyl pyrophosphate levels and potentiate FUra sensitivity (2, 4). Inosine has also been shown to potentiate FUra sensitivity, presumably by elevating ribose 1-phosphate levels (18), but this was not seen in LAZ cells at inosine levels which did not inhibit growth (<10^{-4} M).\textsuperscript{4} Deoxyinosine potentiates FUra sensitivity in LAZ and other cell lines (8, 18), presumably by elevation of deoxyribose 1-phosphate levels.

While we can establish biochemically the route of FUra anabolism, it is not clear to what extent biochemical measurements can predict FUra sensitivity in vivo. In LAZ cells, inhibition of dUrd conversion occurred consistently at 3- to 4-fold higher FUra concentrations than were required to inhibit growth to the same extent (Chart 3). In contrast, the ratio between the FUra concentrations required to inhibit dUrd conversion and growth to the same extent in CEM cells was not constant (possibly because of contributions to growth inhibition by inhibition of RNA metabolism). However, it was lowest at high levels of inhibition (>90%) where approximately 4- to 6-fold higher FUra concentrations gave the same inhibition of dUrd conversion as of growth (Chart 3). If the FUra concentration giving >90% inhibition of dUrd conversion were universally 3- to 6-fold greater than that giving the same inhibition of growth, determination of that concentration might give an indication of sensitivity in vivo for human tissues. However, a further factor which may be involved in FUra sensitivity in vivo is the rate of recovery from thymidylate synthetase inhibition, since this has been shown to vary in different cell types (24, 31). The free FdUMP levels measured in LAZ and CEM cells at >90% inhibition of dUrd conversion were reasonably similar (Tables 5 and 6), but it is unlikely that the level of free FdUMP will generally be indicative of the degree of inhibition of dUrd conversion, since other factors such as the cellular folate status (8, 21, 43) and the ability to elevate dUMP pools (7, 31) will contribute to determining both the amount of FdUMP bound to thymidylate synthetase and the reversibility of this binding.

FUra sensitivity in vivo can also result from inhibition of RNA metabolism. Recent studies with cultured cell lines have indicated that the amount of FUra incorporated into RNA can be correlated with RNA-mediated cytotoxicity (25, 34); thus, this may also prove to be a guide to FUra sensitivity in vivo. Determination of the actual sensitivity to and mechanism of growth inhibition by FUra in different human tissues is obviously a difficult procedure, but at least with a knowledge of the route of FUra metabolism one may be able to selectively modulate FUra action by appropriate manipulation of levels of rate-limiting cosubstrates or of rescue agents.

ACKNOWLEDGMENTS

The authors would like to thank Marlen Dyne and Wanda Deveski for their excellent assistance with the experiments and Professor M. H. N. Tattersall for helpful discussions.

\textsuperscript{4} A. A. Piper, unpublished observations.

REFERENCES


29. Minowada, J. Markers of human leukemia-lymphoma cell lines reflect hae-
Biochemical Basis for the Differential Sensitivity of Human T- and B-Lymphocyte Lines to 5-Fluorouracil

Anita A. Piper and Richard M. Fox


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/42/9/3753

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/42/9/3753. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.