Mechanism of Deoxycytidine Rescue of Thymidine Toxicity in Human T-Leukemic Lymphocytes

Richard M. Fox, Edith H. Tripp, and Martin H. N. Tattersall

Ludwig Institute for Cancer Research, University of Sydney, Sydney, New South Wales, 2006 Australia

ABSTRACT

Cultured malignant human lymphocytes are highly sensitive to growth inhibition by thymidine (50% inhibitory dose = $10^{-5}$ M). Growth inhibition reflects sustained elevation of the deoxythymidine 5'-triphosphate pool associated with secondary elevation of the deoxyguanosine 5'-triphosphate pool and reduction in the deoxycytidine 5'-triphosphate (dCTP) pool. Deoxycytidine was capable of partially reversing thymidine growth inhibition at a concentration of 0.5 μM, and growth recovery was virtually complete at 8 μM. The dCTP pool remained depressed until growth inhibition reversal by deoxycytidine was complete, and at a higher concentration of deoxycytidine the dCTP rose above control levels, but the deoxythymidine 5'-triphosphate and deoxyguanosine 5'-triphosphate pools remained elevated.

These results support the view that thymidine growth inhibition induces a critical deficiency of dCTP via allosteric inhibition of ribonucleotide reductase rather than inhibiting DNA replication directly by elevated deoxythymidine 5'-triphosphate or deoxyguanosine 5'-triphosphate pools.

INTRODUCTION

dThd, at mM levels, will inhibit growth of most cultured mammalian cells. This toxic effect is believed to reflect a sustained increase in the dTTP pool, which allosterically affects ribonucleotide reductase. This results in increased levels of dGTP and reduction in the level of dCTP. The inhibition of cell growth by dThd is reversed by dCyd which increases the dCTP pool, suggesting that dCTP concentration is critical in controlling DNA synthesis (1, 6, 8, 9).

An alternative suggestion has been that dNTP pools may react with a regulatory protein for DNA polymerase, modulating DNA replication. Studies using this regulatory protein isolated from calf thymus have shown that, in the presence of this protein, the activity of DNA polymerase α is inhibited by dGTP in a concentration-dependent manner. It was proposed that physiologically or pharmacologically induced increases in dNTP pools could inhibit DNA synthesis by this mechanism (5, 12, 15).

Recently, it has been observed that the growth of cultured human leukemic lymphoblasts of T- and null-cell origin is inhibited by concentrations of dThd some 2 orders of magnitude below mM levels. The mechanisms of this increased sensitivity appear to reflect a reduced catabolism of deoxynucleosides and/or deoxynucleotides, which allows these cells to sustain an excessive accumulation of dTTP at low concentrations of exogenous dThd (2, 4, 17).

In this paper, we report a study of dCyd reversal of dThd growth inhibition in a cultured human leukemic T-cell line highly sensitive to dThd. We have correlated changes in intracellular dNTP during growth reversal and demonstrate that elevated dTTP pools appear to inhibit cell growth via an allosteric effect on ribonucleotide reductase rather than having a direct effect on the DNA replicating process.

MATERIALS AND METHODS

Cell Lines and Culture. The cell lines studied were: (a) CCRF-CEM, derived from a patient with acute lymphoblastic leukemia and characterized as a T-cell type (7); and (b) LAZ-007, an Epstein-Barr virally transformed lymphocyte with B-cell surface markers. These lines were generously provided by Dr. H. Lazarus (Sidney Farber Cancer Institute, Boston, Mass.). The cells, in suspension culture, were grown in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% fetal calf serum. The lines had similar doubling times (~ 24 hr).

Isotopes and Chemicals. 3H-Labeled dNTP (dTTP, dCTP, dATP, and dGTP) were purchased from The Radiochemical Centre, Amersham, England. Unlabeled dTTP, dCTP, dATP, and dGTP were purchased from Sigma Chemical Co., St. Louis, Mo, and from P. L. Biochemicals, Milwaukee, Wis. Micrococcus luteus DNA polymerase and templates for the DNA polymerase assay for dNTP, poly(deoxyadenylate-deoxythymidyate) and poly(deoxyinosinate-deoxycytidylate) were purchased from Miles Laboratories, Elkhart, Ind. Unlabeled dCyd and dThd were obtained from Calbiochem, La Jolla, Calif.

Deoxynucleoside Inhibition of Cell Growth. These were performed as described previously by us (4).

DNA Polymerase Assay for dNTP. This was performed as described previously by us except that cells were extracted with 60% ethanol rather than with 60% methanol (13). This assay is a modification of the method described by Solter and Handschumacher (11). Results are the means of duplicate assays.

RESULTS

Deoxynucleoside Sensitivity of the T-Cell Line. We have previously reported this cell line to be highly sensitive to dThd (50% inhibitory dose, 30 μM) (4), and these cells were also found to be sensitive to deoxyguanosine with lesser degrees of sensitivity to deoxyadenosine and dCyd. The T-cell is more sensitive to these nucleosides than is the Epstein-Barr virus-transformed B-lymphocyte line (Table 1).

Influence of dThd on dNTP Pools of the Sensitive T-Cell Line. The dThd-sensitive T-cell line (CEM) and the dThd-re-
sistant B-cell line (LAZ-007) were incubated with dThd (60 μM), and at 1 and 48 hr the dNTP pools were assayed. The sensitive T-cell line shows a prompt elevation in the dTTP pool at 1 hr which is sustained at 48 hr. This is accompanied by a marked although lesser rise in the dGTP pool at 1 hr and sustained until at least 48 hr, while the dATP level falls at 1 hr, rising to just above control levels at 48 hr. By contrast, the dCTP pool is depressed at 1 hr and remains depressed at 48 hr (Table 2).

The changes in dNTP levels in the resistant B-cell line (LAZ-007) are less marked. A slight rise in the dTTP pool occurred at 1 hr, but the level returned to normal at 48 hr.

**Rescue of dThd Growth Inhibition by dCyd.** dCyd alone has no effect on the growth of the dThd-sensitive cell line CEM at concentrations of <0.1 mM (Table 1). The effect of simultaneous addition of dCyd (3 μM to 0.1 mM) on dThd (60 μM) growth inhibition was examined. This concentration of dThd suppressed growth to 32% of control, but 3 μM dCyd reversed this inhibition to 82% of control growth, and higher doses (>10 μM dCyd) completely restored growth.

**Influence of dCyd Reversal of dThd Growth Inhibition on dNTP Pools.** dCyd alone at growth noninhibitory doses (10 μM to 0.1 mM) led to 2- to 3-fold elevation of the dCTP, dTTP, and dGTP pools and reduction of the dATP to 66 to 70% of control levels (Table 3). dThd alone caused a marked elevation of the dCTP and dGTP pools, little change in the dATP pool, and reduction in the dCTP pool to 40% of control value (Table 4). The lowest level of dCyd used in this experiment (3 μM) partially reversed dThd growth inhibition without significantly altering dNTP levels, and in particular the dCTP pool remained low. Higher concentrations of dCyd completely reversed growth inhibition and led to elevation of dCTP above control, but only slightly lowered the highly elevated dTTP and dGTP pools.

Despite rescue of growth, the dATP pool fell to =50% of control, a finding similar to that seen after incubation with dCyd alone (Table 3).

The finding that dCyd (3 μM) could partially reverse dThd growth inhibition, without altering dNTP pools, in particular without elevating the dCTP pool was surprising. This phenomenon was therefore examined in more detail. Cells were incubated with dThd (60 μM), and a concentration range of dCyd that was capable of giving minimal to complete reversal of dThd toxicity was used (0.5 to 16 μM dCyd). The dTTP and dCTP pools were assayed at 1 and 48 hr (Chart 1). At 0.5 μM dCyd, the dThd-inhibited growth was reversed from 26% of control to 37% with progressive reversal to 94% at 16 μM dCyd.

In the cells treated with dThd without dCyd rescue, the dCTP pool had fallen to 51% at 1 hr and 44% at 48 hr. At 1 hr, all doses of dCyd used (0.5 to 16 μM) elevated the dCTP pool to normal or supranormal levels (116 to 168% of control). However, at 48 hr, the dCTP pool did not reach control levels until 8 μM dCyd was used, despite the fact that lower concentrations of dCyd were partially reversing growth inhibition. At ≥8 μM dCyd, and dCTP rose 2-fold of control levels.

The dTTP pool in the cells treated with dThd alone was markedly elevated at 1 (760%) and 48 (1069%) hr. In cells reversed dThd growth inhibition without significantly altering dNTP levels, and in particular the dCTP pool remained low. Higher concentrations of dCyd completely reversed growth inhibition and led to elevation of dCTP above control, but only slightly lowered the highly elevated dTTP and dGTP pools.

Despite rescue of growth, the dATP pool fell to =50% of control, a finding similar to that seen after incubation with dCyd alone (Table 3).

The finding that dCyd (3 μM) could partially reverse dThd growth inhibition, without altering dNTP pools, in particular without elevating the dCTP pool was surprising. This phenomenon was therefore examined in more detail. Cells were incubated with dThd (60 μM), and a concentration range of dCyd that was capable of giving minimal to complete reversal of dThd toxicity was used (0.5 to 16 μM dCyd). The dTTP and dCTP pools were assayed at 1 and 48 hr (Chart 1). At 0.5 μM dCyd, the dThd-inhibited growth was reversed from 26% of control growth rate to 37% with progressive reversal to 94% at 16 μM dCyd.

In the cells treated with dThd without dCyd rescue, the dCTP pool had fallen to 51% at 1 hr and 44% at 48 hr. At 1 hr, all doses of dCyd used (0.5 to 16 μM) elevated the dCTP pool to normal or supranormal levels (116 to 168% of control). However, at 48 hr, the dCTP pool did not reach control levels until 8 μM dCyd was used, despite the fact that lower concentrations of dCyd were partially reversing growth inhibition. At ≥8 μM dCyd, and dCTP rose 2-fold of control levels.

The dTTP pool in the cells treated with dThd alone was markedly elevated at 1 (760%) and 48 (1069%) hr. In cells reversed dThd growth inhibition without significantly altering dNTP levels, and in particular the dCTP pool remained low. Higher concentrations of dCyd completely reversed growth inhibition and led to elevation of dCTP above control, but only slightly lowered the highly elevated dTTP and dGTP pools.

Despite rescue of growth, the dATP pool fell to =50% of control, a finding similar to that seen after incubation with dCyd alone (Table 3).

The finding that dCyd (3 μM) could partially reverse dThd growth inhibition, without altering dNTP pools, in particular without elevating the dCTP pool was surprising. This phenomenon was therefore examined in more detail. Cells were incubated with dThd (60 μM), and a concentration range of dCyd that was capable of giving minimal to complete reversal of dThd toxicity was used (0.5 to 16 μM dCyd). The dTTP and dCTP pools were assayed at 1 and 48 hr (Chart 1). At 0.5 μM dCyd, the dThd-inhibited growth was reversed from 26% of control growth rate to 37% with progressive reversal to 94% at 16 μM dCyd.

In the cells treated with dThd without dCyd rescue, the dCTP pool had fallen to 51% at 1 hr and 44% at 48 hr. At 1 hr, all doses of dCyd used (0.5 to 16 μM) elevated the dCTP pool to normal or supranormal levels (116 to 168% of control). However, at 48 hr, the dCTP pool did not reach control levels until 8 μM dCyd was used, despite the fact that lower concentrations of dCyd were partially reversing growth inhibition. At ≥8 μM dCyd, and dCTP rose 2-fold of control levels.

The dTTP pool in the cells treated with dThd alone was markedly elevated at 1 (760%) and 48 (1069%) hr. In cells

---

**Table 1**

\(\frac{\text{Deoxynucleoside sensitivity of cultured lymphocytes}}{}\)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>dThd (μM)</th>
<th>Deoxoguanosine (μM)</th>
<th>Deoxyadenosine (μM)</th>
<th>dCyd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (ALL)² CCRF-CEM</td>
<td>30</td>
<td>20</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>B (Epstein-Barr virus-transformed) LAZ-007</td>
<td>2</td>
<td>2</td>
<td>&gt;3</td>
<td>&gt;3</td>
</tr>
</tbody>
</table>

* ALL, acute lymphoblastic leukemia.

**Table 2**

\(\frac{\text{Influence of dThd incubation on dNTP pool levels in cultured lymphocytes}}{}\)

<table>
<thead>
<tr>
<th>Experiment CCRF-CEM</th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>dTTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>dThd alone (60 μM)</td>
<td>98</td>
<td>40</td>
<td>825</td>
<td>1520</td>
</tr>
<tr>
<td>dThd (60 μM) + dCyd at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. 3 μM</td>
<td>103</td>
<td>24</td>
<td>712</td>
<td>1250</td>
</tr>
<tr>
<td>II. 10 μM</td>
<td>53</td>
<td>346</td>
<td>450</td>
<td>523</td>
</tr>
<tr>
<td>III. 30 μM</td>
<td>49</td>
<td>248</td>
<td>687</td>
<td>869</td>
</tr>
<tr>
<td>IV. 0.1 mM</td>
<td>59</td>
<td>355</td>
<td>442</td>
<td>923</td>
</tr>
</tbody>
</table>

* ALL, acute lymphoblastic leukemia.

**Table 3**

\(\frac{\text{Rescue of dThd Toxicity}}{}\)

<table>
<thead>
<tr>
<th>% of control levels</th>
<th>10 μM dCyd</th>
<th>0.1 mM dCyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>70</td>
<td>66</td>
</tr>
<tr>
<td>dCTP</td>
<td>302</td>
<td>284</td>
</tr>
<tr>
<td>dGTP</td>
<td>285</td>
<td>180</td>
</tr>
<tr>
<td>dTTP</td>
<td>265</td>
<td>243</td>
</tr>
</tbody>
</table>

**Table 4**

\(\frac{\text{Influence of dCyd reversal of dThd growth inhibition of T (ALL)² CCRF-CEM cell dNTP pools}}{}\)

<table>
<thead>
<tr>
<th>% of control values</th>
<th>Experiment CCRF-CEM</th>
<th>dATP</th>
<th>dCTP</th>
<th>dGTP</th>
<th>dTTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>dThd alone (60 μM)</td>
<td>98</td>
<td>40</td>
<td>825</td>
<td>1520</td>
<td></td>
</tr>
<tr>
<td>dThd (60 μM) + dCyd at:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. 3 μM</td>
<td>103</td>
<td>24</td>
<td>712</td>
<td>1250</td>
<td></td>
</tr>
<tr>
<td>II. 10 μM</td>
<td>53</td>
<td>346</td>
<td>450</td>
<td>523</td>
<td></td>
</tr>
<tr>
<td>III. 30 μM</td>
<td>49</td>
<td>248</td>
<td>687</td>
<td>869</td>
<td></td>
</tr>
<tr>
<td>IV. 0.1 mM</td>
<td>59</td>
<td>355</td>
<td>442</td>
<td>923</td>
<td></td>
</tr>
</tbody>
</table>

* ALL, acute lymphoblastic leukemia.
exposed to dThd and dCyd, the dTTP pools were slightly lower at 1 hr from 560 to 384%); but at 48 hr they were higher than in the dThd-alone-treated cells at low doses of dCyd (2483 to 3448%); but at higher doses of dCyd, the dTTP levels were similar to those in cells not rescued with dCyd (Chart 2).

Grindey et al consider the fall in dCTP concentration following exposure to dThd to be too small to account for growth inhibition. They postulate that this increase in the other dNTP pools may be partially responsible for the growth inhibition (5). A potential mechanism for this has been cited, invoking a regulatory protein for DNA polymerase α which has been isolated recently from calf thymus. In the presence of this protein, activity of DNA polymerase α is inhibited by dGTP in a concentration-dependent manner (12). They postulate that physiologically or pharmacologically induced increases in dNTP pools would be capable of inhibiting DNA synthesis by this mechanism.

In studying dCyd reversal of dThd growth inhibition of the lowest dose of dCyd, we initially used (3 μM) partly reversed dThd growth inhibition without elevating the dCTP pool. This paradoxical finding was further examined, and lower doses of dCyd were used in rescue experiments. It was found that 0.5 μM dCyd would partially reverse the inhibition of 60 μM dThd, and this reversal was virtually complete (=95% of control growth) at 8 to 16 μM dCyd. The dCTP pool remained low until growth inhibition was reversed entirely, and at this point the dCTP pool exceeded control levels by 2-fold, but the dTTP and dGTP pools fell to 3 to 4 times the control levels.

These observations suggest that the mechanism of dThd inhibition in the sensitive leukemia lines and relatively resistant mammalian cell lines, i.e., dNTP pool imbalance via feedback inhibition of ribonucleotide reductase, is identical.

The changes in dNTP pools following incubation with dThd appear to be explained by the known in vitro allosteric properties of ribonucleotide reductase. It is believed that the increased cellular content of dTTP following exposure to dThd inhibits the reduction of CDP to dCDP, thereby reducing the dCTP pool. dTTP stimulates the reduction of GDP to dGDP thereby increasing the dGTP pool (1).

An alternate mechanism for dThd-induced growth inhibition has been proposed by Grindey et al. They have studied the changes in dNTP pools in CCRF-CEM cells following exposure to inhibitory concentrations of dThd used after rescue with dCyd (5). At a concentration of 40 μM dThd, which inhibited growth by 73%, the dTTP pool was elevated to 8 times the control levels, and the dGTP and dATP pools were also increased 3- to 5-fold. The dCTP pool was reduced by 42%. On prevention of growth inhibition by dCyd, the dCTP pool increased to control value while the dTTP and dGTP pools fell to 3 to 4 times the control levels.

Grindey et al consider the fall in dCTP concentration following exposure to dThd to be too small to account for growth inhibition. They postulate that the increase in the other dNTP pools may be partially responsible for the growth inhibition (5). A potential mechanism for this has been cited, invoking a regulatory protein for DNA polymerase α which has been isolated recently from calf thymus. In the presence of this protein, activity of DNA polymerase α is inhibited by dGTP in a concentration-dependent manner (12). They postulate that...
overshoot; i.e., the dCTP provided by salvage kinase from dCyd exceeds that normally produced by the reduction of CDP. These observations support the view that dTDP growth inhibition reflects a critical deficiency of dCTP for DNA replication, despite the fact that the measured dCTP pool is only reduced by ≈50%. One possible explanation is that this residual dCTP pool is unavailable to the replicative DNA polymerase. Although little is known about compartmentalization of dNTP, it appears potentially important. Skoog and Bjursell (10) have shown unequal distribution of dNTP between the cytoplasm and nucleus and that this distribution of dNTP changes during the cell cycle. Furthermore, studies of dNTP incorporation into DNA of isolated cell nuclei have demonstrated a Km of isolated DNA polymerase 10- to 100-fold that of enzyme in the nucleus, suggesting that the enzyme in the cell is in the form of a complex of high affinity (16). Alternatively, DNA replication might require a critical concentration of dCTP which might serve a regulatory function.

Clearly, growth reversal by dCyd occurs with only minimal reduction of the markedly elevated dTTP and dGTP pools. This suggests that excesses of dNTP are not directly inhibitory to DNA replication. Similarly, studies of lysed cell or permeable cell systems incubated with exogenous dNTP demonstrate saturation of incorporation of labeled dNTP into DNA but not inhibition at excess dNTP (3, 14).

ACKNOWLEDGMENTS

We thank S. Knott and Dr. I. Taylor for their expert maintenance of these cell lines and A. Rattenbury for her patient typing of the manuscript.

REFERENCES

Mechanism of Deoxycytidine Rescue of Thymidine Toxicity in Human T-Leukemic Lymphocytes

Richard M. Fox, Edith H. Tripp and Martin H. N. Tattersall


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/40/5/1718

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, contact the AACR Publications Department at permissions@aacr.org.