Role of DNA Replication and Repair in Thymineless Death in *Escherichia coli*

Pamela A. Morganroth and Philip C. Hanawalt


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Thymineless death (TLD), the phenomenon in which exponentially growing cells starved for thymine lose viability, has been researched for over five decades, but the molecular mechanism remains an enigma. Thymine starvation halts DNA synthesis; Maaløe and Hanawalt (9) proposed a connection between stalled replication forks and TLD in *Escherichia coli* after showing that only those cells actively replicating DNA when thymine was removed underwent TLD (8). To test the role of replication in TLD, we examined the impact of various concentrations of hydroxyurea (HU) on the viability of *E. coli* cells starved for thymine. HU specifically inhibits replication by preventing the function of ribonucleoside diphosphate reductase, thus starving the cell for all four deoxynucleotides. At high concentrations, HU also significantly inhibits transcription by an unexplained mechanism, but at low concentrations, the effects of HU appear to be largely limited to inhibition of DNA replication (14).

HU exposure affected TLD in a concentration-dependent manner. The protection against TLD afforded by HU increased with increasing concentrations of HU. The thymine-requiring *E. coli* strain HL813 (all strains used in this study are listed in Table 1) was grown overnight at 37°C in Difco Davis minimal medium containing 0.4% glucose and 0.1% Casamino Acids (DM medium) supplemented with 10 μg/ml thymine. The overnight culture was diluted in the same medium and grown at the same temperature with shaking. During the exponential phase (10^6 to 10^8 cells/ml), cells were collected on a Millipore filter (pore size, 0.45 μm) and washed and resuspended in DM medium without thymine. HU was added to the specified bacterial cultures at the designated final concentrations, and at the indicated time intervals 0.01-ml aliquots were removed, appropriately diluted, and plated on DM agar plates supplemented with 10 μg/ml thymine for the determination of viability. When 200 mM HU was added to thymine-starved cells, viable cell counts remained constant over a 4-h time period (Fig. 1a). However, TLD was observed at lower concentrations of HU, albeit at a slightly lower rate than in the control without HU (Fig. 1b and c). At higher HU concentrations, such as 200 mM, RNA synthesis was greatly reduced (Fig. 2a). Since inhibition of RNA synthesis protects against TLD (4, 6, 10), it is likely that the lack of TLD at 200 mM HU is mostly due to inhibition of RNA synthesis. However, inhibition of DNA synthesis could also be involved in the TLD protection, because DNA synthesis was completely inhibited in the presence of 200 mM HU (Fig. 2b). On the other hand, although DNA synthesis was inhibited more than 90% at the lowest concentration of HU used in the HU/thymine starvation experiments, TLD was at most marginally inhibited (Fig. 1c), which indicates that active DNA replication is either not essential for TLD or is involved in only one of multiple TLD pathways. To measure RNA synthesis, cultures of exponential-phase HL850 (a uracil-requiring strain) were treated with HU and incubated for 10 min, and 0.01-ml duplicate samples were pulse labeled with [5,6-^3H]uridine for 10-min intervals at 37°C. [5,6-^3H]Ur fooled was added at a final concentration of 7.5 μCi/ml. After pulse labeling, macromolecules were immediately precipitated by 2 ml of ice-cold 5% trichloroacetic acid and collected on a Millipore filter (pore size, 0.45 μm) and washed with distilled water, and radioactivity was measured by scintillation counting. DNA synthesis was measured by continuous labeling with [methyl-^3H]thymine added at a final concentration of 10 μCi/ml to cultures of exponential-phase HL813 at 37°C. Label was added 10 min after HU treatment, and the

### Table 1. *E. coli* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL353, DM952thy−</td>
<td>D. Mount</td>
<td>thyA deo lac</td>
</tr>
<tr>
<td>HL354, DM953thy−</td>
<td>D. Mount</td>
<td>lexA3 thyA deo lac</td>
</tr>
<tr>
<td>HL313, CGSC6411</td>
<td>Coli Genetic Stock Collection</td>
<td>thyA715 deo lac</td>
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<td>HL850</td>
<td>C. Gross</td>
<td>rph-1</td>
</tr>
<tr>
<td>HL930</td>
<td>J. Courcelle</td>
<td>rph-1</td>
</tr>
<tr>
<td>HL952</td>
<td>J. Courcelle</td>
<td>lac− rrnD−rrnE IN(rph1−)</td>
</tr>
</tbody>
</table>

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* All strains are *E. coli* K-12 F−, with no plasmids.
method used for RNA labeling was employed to determine incorporated radioactivity.

The HU experiments are also interesting in the context of the unbalanced growth theory, which postulates that continued RNA synthesis in the absence of DNA synthesis during thymine starvation is responsible for TLD (2). At low concentrations of HU, such as 75 mM, unbalanced growth occurred in the presence of thymine because DNA synthesis was inhibited, but RNA synthesis continued at a significant rate. If unbalanced growth were the cause of thymineless death, a similar loss of viability would be expected at low concentrations of HU in the presence of thymine. However, although low HU concentrations were bacteriostatic in the presence of thymine, they were not bactericidal (Fig. 1b and c).

Because TLD stalls DNA replication and is associated with the appearance of damaged DNA (10), the mechanism of cell death might be connected to DNA repair responses such as nucleotide excision repair (NER) or the SOS genomic stress response. NER is a cellular response to DNA damage in which stretches of DNA containing damaged nucleotides are removed and replaced by repair replication, with the undamaged strand used as a template (7). The two subpathways of NER, global genomic repair and transcription-coupled repair (which selectively operates on the transcribed strand of expressed genes), both require the UvrA protein (15); thus, in order to determine whether either pathway plays a role in TLD, we characterized TLD in a uvrA mutant by the thymine starvation method used in the HU experiments. The TLD curve of the uvrA mutant HL952 did not differ significantly from that of the isogenic uvrA+ parent strain HL930 (Fig. 3), suggesting that...
neither NER pathway plays an important role in TLD.

The SOS response is a response to replication fork arrest that involves an up-regulation of over three dozen genes controlled by RecA and LexA (3). By stalling the DNA replication fork, thymine starvation induces the SOS response (1, 11); therefore, TLD may be attributable to an inducible killing factor that is part of the genomic stress response. We examined the impact on TLD of a lexA3 mutation, which prevents SOS induction by producing a noncleavable LexA3 protein. When starved for thymine (by the same method as in the HU experiments), the lexA3 mutant HL354 lost viability at a rate nearly identical to that of the isogenic lexA+ parent strain HL353 (Fig. 4). This experiment demonstrates that TLD is not inhibited in the absence of SOS induction and thus could not be due to an inducible killing factor controlled by the LexA/RecA system. Furthermore, because the lexA3 mutant did not show increased or decreased sensitivity to TLD, DNA repair and recombination mechanisms associated with the SOS response do not appear to contribute significantly to the survival or killing of thymine-starved cells.

Although TLD cannot be attributed to an inducible killing factor that is controlled by the LexA/RecA-dependent SOS response, other transcriptional changes that occur as a result of thymine starvation may be responsible for the lethal event. One recent theory, proposed by Sat et al. (13), attributes TLD to the mazEF system, a suicide addiction module in the E. coli chromosome that consists of the stable MazF toxin and the unstable MazE antitoxin. E. coli cells are addicted to the labile antitoxin because they require continuous synthesis of this antitoxin to prevent the toxin from inducing cell suicide. Sat et al. reported that the mazEF suicide system is activated by thymine starvation-induced transcriptional changes and have suggested that this built-in cell death system is responsible for TLD. Although the mazEF theory of TLD has attracted significant attention over the past few years, it cannot account for the phenomenon of TLD. One weakness of the mazEF theory is that the two studies attempting to quantify the resistance to TLD caused by a deletion of mazEF produced conflicting results (5, 13). Both studies used trimethoprim for thymine starvation and compared E. coli harboring a mazEF deletion to parental controls, but while Sat et al. (13) reported a nearly 100-fold survival increase in their mazEF deletion mutant, Godoy et al. (5) observed only a 4-fold increase in survival in one mazEF strain and a 5-fold increase in another. A more significant problem with the mazEF theory is that it is inconsistent with the well-documented resistance to TLD when transcription is inhibited (4, 6, 10). In contrast, the mazEF system causes cell death whenever transcription is inhibited, because the labile MazE is degraded but not replaced by new synthesis, allowing the stable MazF toxin to kill the cell. Sat et al. demonstrated that antibiotics inhibiting transcription and translation, including rifampin and chloramphenicol, cause cell death through the mazEF system (12). If the mazEF system were the cause of TLD, inhibition of transcription with rifampin during thymine starvation would be expected to enhance killing. Conversely, as has been demonstrated in numerous other studies (4, 6, 10), as well as in the HU data presented in this paper, inhibition of transcription with such agents prevents TLD. TLD appears to be a complex, multifaceted process, and the mazEF system may account for one pathway of death triggered by thymine starvation, but the suicide module is not sufficient to account for TLD.

The data presented in this paper clarify the role of DNA replication in the lethal event of TLD and also rule out several mechanistic explanations of the phenomenon. Novel experiments with HU reveal that active DNA synthesis is not a requirement for the major pathway of TLD. The HU experiments and thymine starvation in DNA repair mutants rule out unbalanced growth, the SOS response, and nucleotide excision repair as causal factors of TLD.

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References