Thioguanine-induced S and G\textsubscript{2} Blocks and Their Significance to the Mechanism of Cytotoxicity

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ABSTRACT

The delayed cytotoxic effect of 6-thioguanine (TG) was studied using L1210 mouse leukemic cells in culture. The cell cycle distribution of a population treated continuously with 10^{-5} M TG was compared to that of control cells using flow cytometric analysis. The TG-treated cells had an increase in the fraction of the population in G\textsubscript{2}-M, a decrease in G\textsubscript{1}, and a constant level in S phase. However, the [methyl-\textsuperscript{3}H]thymidine-labeling index decreased dramatically during TG treatment. Thus, it appeared that some cells were arrested in S phase and that G\textsubscript{1} cells did not enter S phase, due to failure to synthesize DNA. To examine the importance of the G\textsubscript{2} and S cell progression blocks, cells were exposed to a lethal treatment of 10^{-5} M TG for 12 hr and returned to normal medium. Under these conditions, the fraction of the population in both S and G\textsubscript{1} decreased, and nearly one-half of the cells accumulated in G\textsubscript{2} by 60 hr after TG addition, compared to a G\textsubscript{2} fraction of less than one-tenth for the control cells. These results showed that the delayed cytotoxic effect of TG was associated with a cell progression block in the second G\textsubscript{2} phase after TG addition, whereas the retention of cells in S phase appeared to be due to readily reversible secondary effects of TG.

INTRODUCTION

TG\textsuperscript{3} and MP have been shown to have several biochemical activities in cells, including inhibition of purine de novo synthesis (4, 7, 10) and incorporation into nucleic acids as thioguanylate (3, 6, 11). However, the problem of determining which of these biochemical activities is responsible for the cytotoxic effects of TG has been approached comparatively recently (8, 12). The natural nucleotide pool sizes were studied in human epidermoid carcinoma (H.Ep. No. 2) cells treated with TG (8) and in L5178Y cells treated with MP or TG (12). In both studies, the ATP and GTP pool sizes were reduced, but the timing of these effects did not correspond to that of cell growth inhibition (12) or loss of viability (8). Also, 6-methyl-mercaptopurine riboside caused even more pronounced effects on cellular nucleotides than MP or TG did but did not cause loss of the capacity for cell division (8, 12). Therefore, both groups of workers concluded that inhibition of purine de novo synthesis was not the primary mechanism of MP- or TG-induced cytotoxicity. On the other hand, selective inhibition of DNA synthesis during the period of treatment offered partial protection against the cytotoxic effects of MP (8, 12) or TG (8), but selective inhibition of RNA synthesis did not (8). These observations strongly suggest that incorporation into DNA is responsible for the cytotoxic effects of TG and MP. Nevertheless, this point is not yet completely resolved because the following finding of Bieber et al. (3) is still unexplained. A line of adenocarcinoma 755 that was resistant to MP in vivo incorporated \textsuperscript{35}S from \textsuperscript{35}SMP into DNA at least as well as did the sensitive tumor line.

The expression of cytotoxicity due to TG, MP, or TdGuo is delayed (12, 13). Barranco and Humphrey (1) reported that Chinese hamster cells exposed in S phase to a lethal concentration of TdGuo continued to progress through the cell cycle and divide before being arrested. These authors concluded that TdGuo does not interfere with the completion of DNA synthesis but rather is incorporated as readily as is deoxyguanosine, with the consequence that subsequent transcription or replication of this TG-substituted DNA is impaired (1). Therefore, we have investigated the cell cycle perturbations in cell populations treated with TG, using flow cytometry, and related these effects to the TG-induced killing of cells. We have distinguished 2 effects: (a) retention of cells in S phase with a block that prevents entry of additional cells into S phase. This effect is readily reversible (preventable) upon drug removal and is therefore considered to be secondary to the mechanism of cell killing; (b) arrest of cells in G\textsubscript{2} phase. This effect is not reversible and is therefore considered to be associated with the primary cause of cell death.

MATERIALS AND METHODS

Cell Culture. L1210 mouse leukemic cells were grown in Fischer's medium with 10% horse serum as described previously (14). The control population-doubling time was 12 hr. For survival determinations, drug-treated cells were separated from the drug-containing medium by centrifugation, washed with fresh, warm medium, and evaluated by growth curve analysis or colony formation in soft agar (14). When determining colony formation, 9 replicate tubes for each data point were incubated for 12 to 14 days. Control colony-forming efficiency was 50 to 75%.

Autoradiography. Cells (10^6/ml) were incubated with TG for various time periods, and the [\textsuperscript{3}H]dThd labeling index was evaluated by autoradiography. [\textsuperscript{3}H]dThd (20 Ci/mmol, 1 μCi/ml) was added to the culture (5 ml) for 15 min; the cells were separated from the medium by centrifugation, resuspended in 0.5 ml of half-strength PBS (0.0125 M KH\textsubscript{2}PO\textsubscript{4}, 0.05 M NaCl, pH 7.6) and fixed with 10 ml ethanol:acetic acid (3:1) for 20 min. The cells were sedimented by centrifugation, resuspended...
in 0.5 ml ethanol:acetic acid (3:1), dropped onto slides, and air dried overnight. For autoradiography, the slides were washed with water and coated with liquid photographic emulsion NTB2 or NTB3 (Eastman Kodak Co., Rochester, N. Y.). The slides were stored in light-tight boxes in the refrigerator for an exposure period of 4 days and developed. The \[^{3}H\]dThd-labeling in 0.5 ml ethanol:acetic acid (3:1), dropped onto slides, and air dried overnight. For autoradiography, the slides were washed with water and coated with liquid photographic emulsion NTB2 or NTB3 (Eastman Kodak Co., Rochester, N. Y.). The slides were stored in light-tight boxes in the refrigerator for an exposure period of 4 days and developed. The \[^{3}H\]dThd-labeling

Flow Cytometry. The cells were separated from the medium by centrifugation and washed with PBS. The cells (1 to 2 x 10^6) were resuspended in 1 ml of PBS and fixed with 25 ml of 10% buffered formalin [10% formalin (3.7% formaldehyde final concentration) in PBS] which was added while the suspension was stirred vigorously with a vortex mixer. The cells were fixed at 4°C for at least 24 hr and then stained in suspension using an acriflavin:feulgen technique (adapted from the technique of Culling and Vasser (5)). The population distribution of cellular DNA contents was obtained using a Becton-Dickinson FACS III flow cytometer, and the fraction of cells in each phase of the cell cycle was derived from the data by the simulation analysis procedures of Roti Roti et al.4,5

Chemicals. The sample of TG used for these studies was supplied through the courtesy of Professor L. B. Townsend, University of Utah, currently at the University of Michigan.

RESULTS

Continuous treatment of L1210 cells in culture with growth-inhibiting concentrations of TG resulted in a period of reduced growth rate followed by a total cessation of growth (Chart 1). The plateau level attained depended upon the concentration of TG in the cultures. With 10^{-4} M TG, the cell number reached 1.8 times the initial number; with 10^{-5} M TG, the cell number reached about 2.5 times the initial number; and with 10^{-6} M TG, the cell number reached about 4 times the initial number. Thus, cells divided once or more times in the presence of TG before being arrested. The duration of TG treatment required to cause the total cessation of growth or cell killing was determined next. Cells were treated for 3 hr with various concentrations of TG, and their viability was assayed by determining their colony-forming efficiency. When cells were treated with either 10^{-4} M or 10^{-5} M TG, the same fraction, ~63%, of the cells was killed, whereas 10^{-6} M killed only 39% (Table 1).

After longer treatment times (5 hr or more), TG caused total cessation of growth in suspension cultures (Chart 2). In these experiments, the cells were treated with 10^{-4} or 10^{-5} M TG for various times (5 to 24 hr). The TG was then removed, and the subsequent growth of the cells was evaluated by growth curve analysis. In all of these cases, the total cell number in the cultures reached a plateau level, which depended upon the drug concentration and time of treatment. The cell number at the plateau level in the TG-treated cultures was always less than one-half of the cell number at the stationary-phase plateau level in control cultures, indicating that the plateau reached in TG-treated cultures was due to the effects of TG. In Chart 2, the ratio of the cell number at the plateau level to the cell number at the beginning of TG treatment was plotted against the time of TG treatment to provide a measurement of the total cell division capacity. When the cells were treated for 12 hr or more with 10^{-5} M TG, they stopped growing at the same plateau level attained under continuous treatment for about 75 hr. The plateau levels were maintained for at least 140 hr after removal of the TG-containing medium, suggesting that most of the cells were killed by the TG treatment.

Confirmation that the establishment of a cell number plateau in these cultures reflected killing of most cells by the TG

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Table 1

Survival assay by colony formation from cells treated with TG

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>TG concentration (M)</th>
<th>Surviving fraction (%) of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10^{-4}</td>
<td>36 (27-47)</td>
</tr>
<tr>
<td>3</td>
<td>10^{-5}</td>
<td>38 (28-49)</td>
</tr>
<tr>
<td>3</td>
<td>10^{-6}</td>
<td>61 (55-70)</td>
</tr>
<tr>
<td>5</td>
<td>10^{-5}</td>
<td>11 (11-11)</td>
</tr>
<tr>
<td>12</td>
<td>10^{-5}</td>
<td>0.35 (0.21-0.47)</td>
</tr>
</tbody>
</table>

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\* J. L. Roti Roti, R. Waki, and O. Blair, manuscript in preparation.

treatment was obtained by soft agar colony formation. Treatment with $10^{-5}$ M TG for 12 hr was sufficient to kill more than 99% of the cells (Table 1). While $10^{-4}$ and $10^{-5}$ M TG showed similar levels of cell killing after 3 hr of treatment (Table 1), there was a difference between the 2 concentrations in the growth inhibition after longer treatment times, as seen in the growth curve experiments (Chart 2). Therefore, it appeared that the additional growth inhibition by $10^{-4}$ M TG compared to $10^{-5}$ M might be due to secondary effects not directly involved in the mechanism of cell killing. On the basis of these observations, 12 hr with $10^{-5}$ M TG was chosen as the minimal lethal treatment to be used in subsequent experiments.

The mechanism of TG-induced cell killing was further investigated by studies of the changes in cell cycle distribution that resulted from treatment with $10^{-5}$ M TG, using flow cytometric analysis and autoradiography. Chart 3A shows typical histograms of DNA content obtained by flow cytometry after various times of continuous treatment with TG. The fractions of the population in the various cell cycle phases were calculated from the histograms by simulation analysis (10). Chart 3B shows the fraction of the population in the G$_1$, S, and G$_2$-M cell cycle stages at various times during continuous treatment with TG. In the first 12 hr, no significant alterations were observed. Between 12 and 36 hr, the fraction of the cells in G$_2$-M increased and the fraction of the cells in G$_1$ decreased, whereas the fraction of the cells in S phase remained nearly constant. Between 36 and 72 hr, the fraction of the population in each cell cycle phase did not change significantly. The increase in the G$_2$-M fraction between 12 and 36 hr represented cells in the G$_2$ phase, since the mitotic index did not increase during treatment with TG but remained at the control level (0.018) or below (data not shown). These results indicated that, after cessation of cell division in the presence of TG, some cells continued to progress through the cycle, leading to a relative depletion of G$_1$ population and to an accumulation of cells in G$_2$ due to arrest in that phase while a nearly normal fraction remained in S phase. Autoradiographic determination of the $[^3H]$dThd-labeling index presented quite a different picture (Chart 4). For the first 36 hr, the fraction of cells labeled with $[^3H]$dThd remained essentially constant, as did the fraction of cells having DNA contents corresponding to S phase. However, at subsequent times, the $[^3H]$dThd-labeling index decreased markedly, reaching about 10% at 72 hr, whereas the flow cytometric data indicated that about 60% of the cells still had DNA contents corresponding to S phase. Taken together, these observations suggested that, during this later time interval, 36 to 72 hr, some cells were arrested in S phase but that no additional cells entered S phase. Cells that were in G$_1$ when this block became effective would then remain in G$_1$. The cells arrested in G$_1$ and S phase evidently did not synthesize DNA (as reflected in the decreased $[^3H]$dThd-labeling index) and...
thus could not progress into G2 where other cells had been arrested. Accordingly, the fraction of cells in the various cell cycle phases remained essentially constant after 36 hr as mentioned above (Chart 3).

The next experiment was designed to determine whether it might be possible to separate these 2 effects of TG, S-phase arrest with G1-S blockade and G2-phase arrest. Treatment for only 12 hr, the minimal time to produce the maximal growth inhibition with $10^{-5}$ M TG (Chart 2), was expected to minimize the possibility of secondary biochemical effects, while still allowing full expression of the cytotoxic action of TG. Chart 5A shows a typical histogram of DNA content obtained by flow cytometric analysis of the cell population after this treatment and a control. As discussed above, treatment for 12 hr with $10^{-5}$ M TG caused no significant change in the cell cycle distribution. After 12 hr, the cells were removed from the TG-containing medium and returned to normal medium. Typical histograms of DNA content for the population 24 and 48 hr later are also shown in Chart 5A. A progressive shift of the population was observed into the region of the histogram corresponding to the G2 content of DNA. Chart 5B summarizes the cell cycle distribution parameters derived from several such experiments. The accumulation of cells in G2-M was quite pronounced, rising from a control value of less than 0.1 of the population to nearly 0.5 at 48 hr after removal of TG. At this time, the mitotic index was less than 0.010, indicating that the increase in the G2-M fraction was due to G2-phase cells, as in the experiment described above. At the same time, the fraction of the population in both G1 and S decreased. These results suggest that, when TG was removed after the lethal 12-hr treatment, most cells progressed through the cycle, completed S phase, and were arrested in G2. Thus, the continuous presence of TG was required for the S-phase block, whereas the TG which was incorporated during the first 12 hr of treatment was sufficient to cause the irreversible G2-phase arrest.

**DISCUSSION**

This study showed that the delayed cytotoxic effect of TG was associated with irreversible block of cell progression in the G2 phase of the cell cycle. The delay of TG cytotoxicity is seen in Chart 1, where L1210 cell division was shown to continue for a time in the presence of TG before stopping. In addition, virtually total cell killing could be caused by 5–12 hr treatment with TG (Table 1; Chart 2). After TG was removed from the cultures, cell division continued for an additional period before the lethal effect caused total cessation of cell division. This type of delayed cytotoxicity has also been reported by Tidd and Paterson (12, 13) and by Parks et al. (9) for L5178Y cells. A lethal treatment of 12 hr with $10^{-5}$ M TG (Table 1) was sufficient to induce G2 arrest (Chart 5). This result suggested that the G2 block might play the major role in the cytotoxic effect of TG, particularly in vivo where the effective exposure time of target cells may be limited. On the other hand, some cells were also retained in S phase during continuous treatment with TG. The observations that the fraction of cells synthesizing DNA dropped off sharply (Chart 4), while the fraction of cells having DNA contents corresponding to S phase remained high (Chart 3), suggested a lack of precursors for DNA synthesis such as would result from inhibition of purine de novo synthesis. The ability of cells to overcome the S-phase block, when TG was removed after 12 hr, was consistent with the suggestion that the S-phase arrest might be due to readily reversible secondary biochemical effects, such as inhibition of purine de novo synthesis. After the 12-hr treatment, the implication would be that purine de novo synthesis was not inhibited to a sufficient extent to cause S-phase retention and that the cells then progressed to G2.

The irreversible G2 arrest has thus been identified in this study as an event of primary importance in TG-induced cell death. The data also provided some information on the possible...
mechanism of induction of the G2 block. Since this block became permanent after comparatively short times of incubation with TG, the mechanism might be related to the substitution of TG for guanine residues in the DNA that has been reported previously (3, 6, 11). When L1210 cells were treated with $10^{-5}$ M TG (Charts 1 and 2), cell number reached a plateau at about 2.5 times the initial number. This observation was interpreted as representing one division of all cells plus a second division of some cells. This interpretation is consistent with the observations of Barranco and Humphrey (1) who used synchronized Chinese hamster ovary cells. They reported that cells were sensitive to the lethal effect of TdGuo only if they were exposed to the drug during S phase and that such lethally treated cells completed one S phase, progressed through G2 at the normal rate, and divided once before being arrested. In this study, exponentially growing L1210 cells were exposed to TG. Therefore, it appeared that cells that were in G1 phase or early to middle S phase (altogether comprising the 75% of the cells that are positioned earliest in cell cycle) at the time TG was added to the cultures were effectively exposed in S phase, divided once, and were arrested. However, the remaining cells that were in G2 phase or late S phase (altogether comprising the 25% of the cells that are positioned latest in the cell cycle) at the time TG was added to the cultures divided once and then were effectively exposed in S phase. These cells divided a second time before being arrested. Thus, all cells appear to complete one G2 phase and one division after being effectively exposed to TG during S phase, as in the study of Barranco and Humphrey (1). In this study, we have further showed that irreversible arrest of cells lethally treated with TG ($10^{-5}$ M, 12 hr) occurs in the G2 phase of the subsequent cell cycle. Any mechanism that is proposed to explain the cytotoxic effect of TG, therefore, should be consistent with the cell arrest in the second G2 phase after effective S-phase exposure to TG. Thus, interference of TG substitution with subsequent replication of the DNA would not be considered a likely mechanism since the retention of cells in S phase is prevented by removal of the TG, even after a lethal treatment (Chart 5). The observation that cell arrest occurs in the second G2 phase and not the first further suggests that the mechanism may even involve some additional TG substitution in DNA during the second S phase. Possible effects which might cause a block to cell progression in the G2 phase would include inhibition of RNA transcription, production of defective RNA's, or inhibition of chromosomal segregation. The molecular effects of TG substitution in synthetic polymers have been studied by Beikirch et al. (2). They reported that transcription of poly[d(A-C)-d(T-sG)] and poly[d(A-sG)-d(T-C)] by RNA polymerase B from mouse myeloma was inhibited in comparison with the transcription of corresponding polymers containing guanine. This observation would suggest that inhibition of RNA synthesis might play a role in inducing the G2 block that stopped growth of cells treated with TG.

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REFERENCES

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