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CHARACTERIZATION OF THE DNA DAMAGE IN 6-THIOGUANINE-TREATED CELLS

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Abstract—6-Thioguanine (TG) incorporation into DNA has been associated with cytotoxicity and DNA damage in Chinese hamster ovary (CHO) and murine leukemia L1210 cells. According to alkaline elution analysis, single-strand breaks (SSB) occur in both cell types. DNA-protein and interstrand cross-links are prominent features of TG effects in L1210, CEM, and HL-60 but not CHO cells. To assess which DNA strand experiences SSB in CHO cells, the cells were synchronized by growth to confluence (late G_1, S). The cells were then diluted into fresh medium so that they underwent a round of division during a subsequent 16-hr interval. They were treated with TG during this first cell cycle, and mitotic cells were harvested at the end of the first cycle using colcemid. SSB were determined in parental DNA (radiolabeled with thymidine during growth to confluence), TG-containing DNA (radiolabeled with [^{14}C]TG during drug exposure), and daughter DNA (labeled with thymidine during the second cell cycle). SSB occurred in TG-containing DNA late in the second cell cycle after drug exposure and in the DNA synthesized from a TG-DNA template (daughter DNA). This observation is consistent with the known delayed cytotoxicity and chromosomal aberrations seen in CHO cells. The SSB suggest relatively normal elongation of DNA containing TG but altered synthesis and/or ligation from a TG-DNA template. This premise was tested in synchronized CHO cells. The DNA strand incorporating TG elongated naturally; however, DNA elongation was impaired in the cell cycle following TG treatment. The results are consistent with SSB in daughter DNA synthesized from a TG-DNA template due to inability to elongate the newly-synthesized strand.

The cytotoxic properties of 6-thioguanine (TG†) are at least partially due to its incorporation into DNA [1]. Possible consequences of TG incorporation include single-strand breaks (SSB) [2–4] and double-strand breaks (DSB) [3], DNA-protein cross-links (DPC) and interstrand cross-links (ICL) [5], enhanced sister chromatid exchange [6], and overt chromosomal damage [7, 8]. Chromosomal abnormalities have been reported during the G_2 phase of the cell cycle following TG treatment [7]. In a model of the TG-cytosine base pairs that might result from TG incorporation into DNA, Thewalt and Bugg [9] suggested that the bulkier S atom, compared with the exocyclic oxygen of guanine, may lead to a distortion in the sugar-phosphate backbone of double helical nucleic acids that impairs function. We have reported previously TG-altered function of vaccinia viral DNA (i.e. reduced ability to induce thymidine kinase activity during early infection) [10] and in bacterial DNA (reduced transforming activity) [11]. Since these effects of TG may be associated with the physical damage to DNA observed by alkaline elution, we sought to characterize the damage and to ascertain which particular DNA species are damaged. The work presented herein suggests that the predominantly SSB in synchronized CHO cells occur in DNA containing TG and in the DNA synthesized from a TG-DNA template. A preliminary report of this work has been presented [12].

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† Abbreviations: CHO, Chinese hamster ovary; DPC, DNA-protein cross-links; DSB, double-strand breaks; ICL, interstrand cross-links; SeGuR, 6-selenoguanosine; SDS, sodium dodecyl sulfate; SSB, single-strand breaks; TG, 6-thioguanine; and TGR, 6-thioguanosine.

MATERIALS AND METHODS

Cell cultures. CHO cells were grown in McCoy's 5a medium as previously described [13]. Human lymphoblastic cells and murine leukemia L1210 cells were grown in RPMI 1640 medium as suspension cultures containing 10% fetal calf serum, 50 units/mL penicillin, and 5 μg/mL streptomycin. The cells were treated with TG during exponential growth, and control cells were treated in each case with equimolar concentrations of guanine. To synchronize CHO cells, the cells were permitted to grow to confluence, such that most were in late G_1 and yielded a wave of mitoses 16–20 hr after subculture [14] (confirmed by flow microfluorometry). To collect cells in their first mitoses, we removed loose cells from the dishes by vigorous pipetting 16 hr after subculture. Colcemid (0.025 μg/mL) was then added, and the cells were incubated for an additional 3 hr. The accumulated mitotic cells were then selectively detached from the dishes. The mitotic cells were reseeded into fresh medium and permitted to undergo a second round of division. Cells were collected 0, 4, and 24 hr after the colcemid arrest to assess DNA damage by alkaline elution analysis.

Alkaline elution. Cells were harvested by trypsin digestion (CHO cells) or centrifugation. The alkaline elution techniques of Kohn and Grimek-Ewig [15] were used to determine the types of DNA damage.

Polycarbonate filters (2.0 μm pore diameter) were used to assay for SSB, DSB, and ICL, whereas 2.0 μM pore diameter polyvinyl chloride filters were used to assay for DPC. Approximately 5×10^5 cells were applied by gravity flow to each filter in 10 mL of 0.14 M NaCl, 5 mM KCl, 5 mM dextrose and 5 mM $NaHCO_3$. The cells were then lysed with 5 mL of lysing solution (2% SDS, 0.025 M EDTA, pH 10.1, for SSB and ICL; 0.2% sarcosyl, 2 M NaCl, 0.04 M EDTA, pH 10.1, for DPC; 2% SDS, 0.025 M EDTA, pH 9.6–10, proteinase K, 0.5 mg/mL for DSB). The lysates were rinsed with 5 mL of 0.02 M EDTA before elution of the DNA with 0.1 M tetrapropyl ammonium hydroxide plus 0.02 M EDTA (pH 12.1, or in the case of DSB, pH 9.6). Fractions were collected every 90 min and analyzed for radioactivity by liquid scintillation spectrometry. The presence of ICL and DPC was evaluated in cells irradiated with γ-rays from a cesium-source (300 and 3000 rads respectively). Elution profiles were obtained with and without a 30-min treatment of the cells with proteinase K (0.5 mg/mL) in the lysing solution. To account for interfilter variation in DNA retention, all alkaline elution experiments were performed using [^{3}H]-labeled (minimum of 50,000 dpm/filter) control samples and [^{14}C]-labeled (minimum of 10,000 dpm/filter) treated samples. Radioactive guanine and TG can be used to label cellular DNA for alkaline elution analysis because all radioactivity collected during alkaline elution was due to incorporation into DNA, rather than RNA. This was established in experiments using [5-^{3}H]uridine and [^{14}C]thymidine, i.e. the lysing solution was adequate to hydrolyze all the cellular RNA prior to fraction collection.

DNA elongation. Effects of TG treatment on DNA elongation were evaluated in synchronized CHO cells using a modification of the above procedure. Cells were treated with TG (3 μM) either during the first cell cycle or during the second cell cycle (following colcemid arrest and reseeding). DNA elongation was determined 4 hr after colcemid arrest by pulse-labeling the cells with [^{3}H]thymidine (1 μCi/mL for 15 min). Following a cold chase with 2.5 μg/mL thymidine, DNA elongation was determined during a subsequent 4-hr interval by alkaline elution.

Determination of TGR in SeGuR. A sample of commercial SeGuR (Sigma Chemical Co., St. Louis, MO; Lot No. 82F-0245) was subjected to quantitative mass spectral analysis for the presence of TGR. Samples were introduced into a Vestec model 201 Dedicated Thermospray LC-MS System (Vestec, Inc., Houston, TX). The amount of TGR was calculated from the ion abundance of mass units 300 and 168 compared with those for SeGuR, i.e. 348, 216, 258, and 136. The presence of TGR in these samples of SeGuR was also confirmed by HPLC analysis using UV absorbance detection at 340 nm and fluorescence detection of the permanganate oxidation product of TGR [16]. Incorporation of the TG from either TGR or SeGuR into the DNA of CHO cells was determined as previously described, with the exception that P1 nuclease (Sigma Chemical) was used to degrade the DNA to its 5′-monophosphates prior to HPLC analysis.

RESULTS

The mechanism of action of TG has been studied extensively in L1210 and CHO cells. In both cell types, DNA damage as assessed by alkaline elution analysis has been correlated with cytotoxicity. The types of damage produced, however, may be different. SSB were readily demonstrated in both cell types (Fig. 1). However, DPC and ICL were prominent effects of the drug in L1210 but not CHO cells. To determine whether this apparent diversity of effects extends to other cell lines, experiments similar to that illustrated in Fig. 1 were performed using two human lymphoblastoid cell lines, HL-60 and CEM (Table 1). DPC were apparent in HL-60 and CEM cells as in the L1210 cells. On the other hand, ICL were readily observed in CEM and L1210 but not HL-60 cells. Cell cloning indicated that the concentration of TG used (3 μM) was approximately equal to its EC_{50} for the 16-hr exposure time in CHO, CEM, and HL-60 cells; however, this concentration was about 100-fold higher than the EC_{50} value (0.03 μM) in L1210 cells.

The above-described experiments used simultaneous TG treatment and thymidine labeling of the DNA; therefore, it was not known whether the DNA that appeared damaged actually contained TG. To address this question, we employed synchronized CHO cells (Fig. 2). In all cases, the CHO cells were exposed to TG (3 μM; the approximate EC_{50} for cell kill) during the first cell cycle. DNA damage was assessed by alkaline elution analysis at the end of the first cycle in cells harvested at mitosis, and at 4- and 24-hr following mitosis. As indicated in Table 2, parental DNA (labeled prior to exposure to TG) was not damaged at any of these times. TG-containing DNA (labeled with [^{14}C]TG during drug exposure) exhibited SSB late (24 hr) but not early (4 hr) in the cell cycle following drug treatment. Damage to DNA synthesized from a TG-DNA template is suggested in that SSB were seen in cells labeled with thymidine during the cell cycle following TG treatment, i.e. DNA retention after 12-hr elution was only 62% of control. These observations suggest that DNA elongation proceeds normally during drug exposure; however, synthesis from a TG-DNA template may not. To determine whether elongation is impeded by TG in a growing strand compared with that synthesized from a TG-DNA template, we used a standard alkaline elution technique either during simultaneous exposure to TG or in the cell cycle following TG treatment in synchronized cells (Fig. 3). As predicted from the late DNA damage observed (Table 2), elongation in a DNA strand that was incorporating TG (i.e. simultaneous exposure and measurement) was not impaired. On the other hand, elongation was delayed markedly in the DNA synthesized from DNA that had already incorporated TG (i.e. treated with TG in the first cell cycle).

The types of DNA damage in TG-containing and daughter DNA were evaluated in synchronized cells treated with TGR and SeGuR as described above for Table 2. Specifically, cells were treated with 3 μM TGR or 35 μM SeGuR during the first cell cycle. The DNA was radiolabeled during the first or second

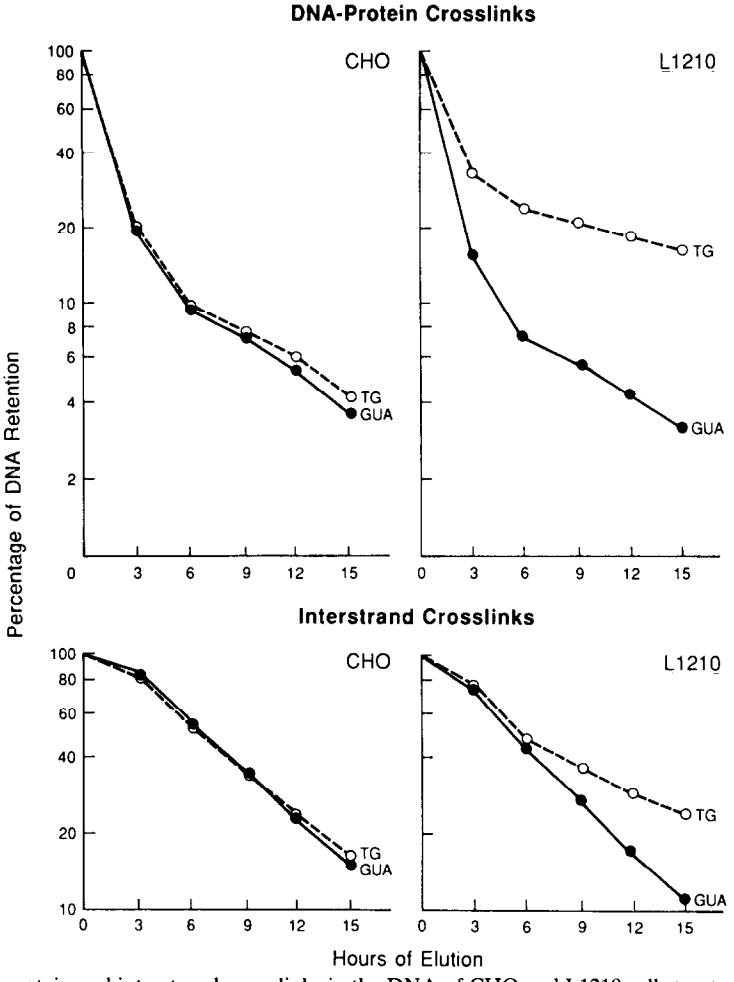

Fig. 1. DNA-protein and interstrand cross-links in the DNA of CHO and L1210 cells treated with TG. CHO or L1210 cells were treated with 3 μM [^{14}C]TG or [^{3}H]Gua (control) for 16 hr as described in Materials and Methods. The cells were then harvested and analyzed for DNA damage by alkaline elution. Aliquots of treated and control samples were subjected to elution on the same filters. The total dpm of ^{14}C and ^{3}H, respectively, applied to the filters was as follows: CHO cells, 11,000 and 87,000; L1210 cells, 55,000 and 286,000.

cell cycle using [^{3}H]thymidine (control, 3 or 35 μM guanosine) or [^{14}C]thymidine (treated). DNA damage was assessed by alkaline elution analysis of aliquots of treated and control cells applied to the same filters as described in Materials and Methods. The results obtained using 35 μM SeGuR were essentially identical to those found with 3 μM TGR (right side of Fig. 4). By mass spectral analysis, the sample of SeGuR used contained approximately 6% TGR. Further, the EC_{50} values for CHO cytotoxicity were virtually identical at these concentrations after correcting for the TGR content in SeGuR (i.e. 2 vs 34 μM respectively). Correspondingly, the incorporation of TG into the DNA of cells treated with these concentrations of TGR and SeGuR was similar (1.5 vs 1.2 nmol/10^9 cells respectively). SSB, but not DSB, were apparent in both TG-containing and daughter DNA (Fig. 4). DPC were also apparent in the higher molecular weight TG-containing DNA, i.e. the treated samples were retained more than control at the later times of alkaline elution. DPC were not obvious in the daughter DNA, i.e. treated samples eluted more rapidly than controls, as anticipated from the SSB observed.

These observations suggest that TG might remain in the DNA of treated cells for at least one cell cycle following treatment. As indicated in Table 3, TG persisted in the nucleic acids of CHO cells for several hours following drug removal, whereas the soluble nucleotide pool was more readily depleted. Thus, delayed properties of the drug may reflect the inability of CHO cells to remove the drug once it has been incorporated into RNA or DNA.

DISCUSSION

Using alkaline and neutral sucrose density gradient analysis, Lee and Sartorelli [3] previously observed both SSB and DSB in L1210 cells treated with high doses of TG. Using their methodology, we have also observed these effects of TG in the DNA from *Bacillus subtilis* (unpublished data). Covey *et al.* [5]

Table 1. Types of DNA damage produced by 6-thioguanine in cultured lymphoblasts*

Type of damage	Cell line	DNA retention (% of control) Treatment protocol† +4 hr	+24 hr
DPC‡	CEM	188 ± 45	248 ± 25§
	L1210	254 ± 86	131 ± 17
	HL-60	331 ± 58§	239 ± 58
ICL‖	CEM	112 ± 14	169 ± 30§
	L1210	163 ± 20§	75 ± 6§
	HL-60	98 ± 1	79 ± 6§

* Cells were grown in tissue culture and treated with 6-TG (3 μM) during exponential, asynchronous growth as described in Materials and Methods. The results shown are the relative retention of DNA in treated versus control samples by alkaline elution analysis (12-hr retention). Mean values ± SE for three or more separate experiments are given.

† The DNA of control cells was labeled with [^{3}H]TdR, and the DNA of treated cells was labeled with [^{14}C]TdR. Treatment with 6-TG was during the interval of labeling (16 hr), and alkaline elution was performed 4 and 24 hr after removal of 6-TG and radiolabeled TdR. The total ^{14}C and ^{3}H applied to the filters ranged from 19,000 to 25,000 and 60,000 to 171,000 dpm respectively.

‡ DPC: DNA protein cross-links. Alkaline elution was performed after treating the cells with 3000 rad as described in Materials and Methods. A value greater than 100 indicates DPC.

§ Significantly different from control, $P \leq 0.05$ (paired t-test).

‖ ICL: interstrand cross-links. Alkaline elution was performed after irradiating the cells with 300 rad. A value greater than 100 is indicative of ICL. A value of less than 100 probably reflects SSB.

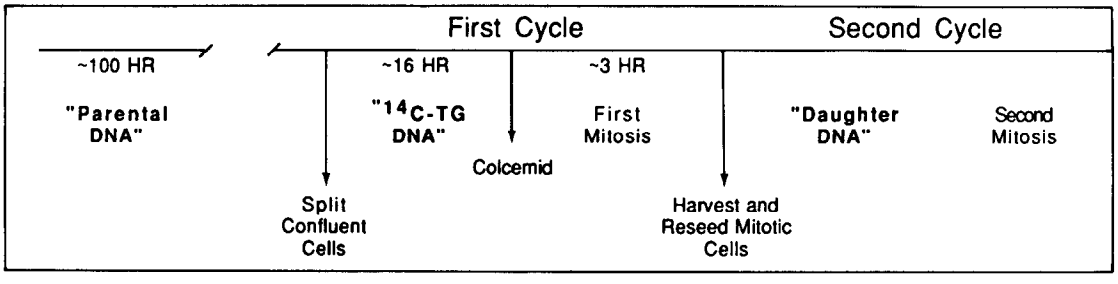

Fig. 2. Scheme for analysis of TG effects in synchronous CHO cells.

reported SSB, DPC and ICL in the DNA from L1210 cells treated with TG; however, Christie *et al.* [2] only observed SSB in the DNA from CHO cells treated with TG. Confirmation that these apparently diverse properties can be reproduced in the same laboratory is given in Fig. 1. Although a 16-hr exposure to 3 μM TG was much more toxic to L1210 cells than to CHO cells, this increased toxicity alone cannot account for the differences in types of DNA damage seen in these cell lines. Specifically, this concentration of TG was approximately equal to its EC_{50} value against CHO, CEM, and HL-60 cells; however, as in L1210 cells, DPC were readily observed in the CEM and HL-60 cells (Table 1). Although the ease with which a particular type of DNA damage is demonstrated by alkaline elution analysis may vary among different cell types, this seems an unlikely explanation for the lack of apparent DPC in TG-treated CHO cells, particularly since DPC have been readily observed via alkaline elution analysis of CHO cells treated with the topoisomerase II inhibitor *m*-AMSA [4′-(9-acridinylamino)methanesulfon - *m* - anisidide] (unpublished observation). In the DNA evaluated for damage in synchronized CHO cells, DPC did appear to occur in the TG-containing but not in daughter DNA (Fig. 4). Whether these apparent differences among cell lines or sources of DNA are truly a function of the effects of the drug or are a function of the ease with which a type of damage can be demonstrated by alkaline elution is not known.

To determine which DNA in TG-treated cells might experience damage, we evaluated SSB in synchronized CHO cells (Table 2). SSB did not occur in the parental DNA (radiolabeled prior to TG exposure). However, SSB were apparent in the DNA labeled with TG or with thymidine in the cell cycle after TG treatment (daughter DNA). Further, the SSB occurred late (24 hr) rather than early (0–4 hr) in the cell cycle after TG treatment. This observation

Table 2. 6-Thioguanine-induced single-strand breaks in the DNA of synchronized Chinese hamster ovary cells*

	DNA retention (% of control)		
Radiolabeled DNA	First mitosis	+4 hr	+24 hr
Parental†	92.5 ± 0.5	100 ± 1	96.6 ± 6.5
TG-containing‡	94.5 ± 1.6	103 ± 4	80.6 ± 4.2
Daughter§			62.0 ± 5.4

* CHO cells were synchronized and treated with TG or guanine as described in Materials and Methods. The values given are the DNA retention values after 12-hr alkaline elution for treated vs control samples. A value of less than 100 is indicative of SSB. The results shown are mean values ± SE for at least three separate experiments. In all cases, aliquots of control and treated cells were subjected to alkaline elution analyses on the same membrane filters, using [^{3}H] (control) and [^{14}C] (treated) labeling of DNA. The total ^{14}C and ^{3}H applied to the filters ranged from 17,000 to 61,000 and 23,000 to 116,000 dpm respectively.

† Cells were grown in the presence of [^{3}H]TdR (control) or [^{14}C]TdR (treated) to confluence as depicted in Fig. 2. They were then exposed to 3 μM guanine (control) or 3 μM TG (treated) during the first cell cycle.

‡ Cells were grown in the presence of 3 μM [^{3}H]guanine (control) or 3 μM [^{14}C]TG (treated) during the first cell cycle, as illustrated in Fig. 2.

§ DNA was labeled during the cell cycle after 3 μM TG exposure during the first cell cycle (see Fig. 2). Prior to cell harvest, the radiolabel was washed and chased for 4 hr with cold TdR.

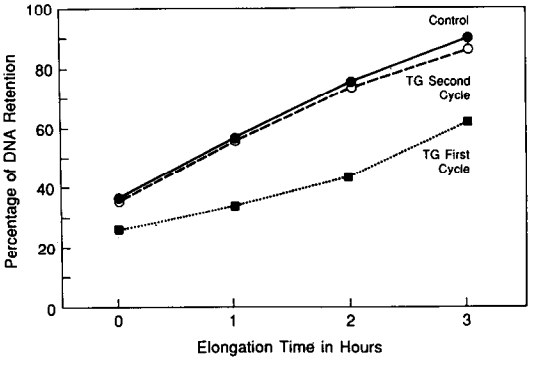

Fig. 3. Inhibition of DNA elongation by TG. Synchronous CHO cells were subjected to TG treatment during the first or second cell cycle as in Fig. 2. DNA elongation was determined beginning 4 hr after collection of mitotic cells at the end of the first cycle. Thus, TG treatment during the second cell cycle represents simultaneous treatment during elongation, whereas TG treatment during the first cell cycle represents sequential treatment. Elongation was determined by alkaline elution analysis following a pulse-chase with [^{3}H]thymidine as described in Materials and Methods. The result shown is representative of two independent experiments. The total dpm × 10^3 applied to the filters was 22.8 ± 9.4 and 22.9 ± 11.1 for TG-treated and control cells respectively (mean ± SE, N = 4).

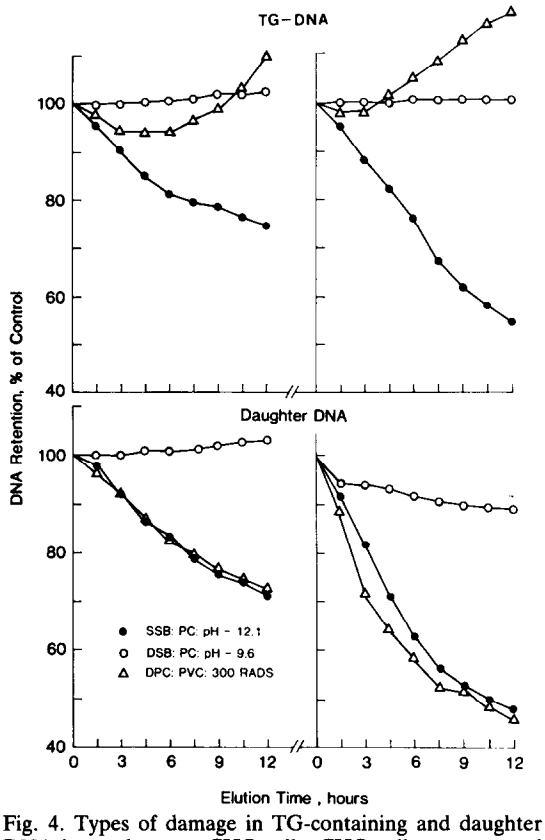

Fig. 4. Types of damage in TG-containing and daughter DNA in synchronous CHO cells. CHO cells were treated with 3 μM TGR according to the protocol outlined in Fig. 2. TG-containing DNA was labeled with [^{14}C]thymidine during the first cell cycle; daughter DNA was labeled with [^{14}C]thymidine during the cell cycle following TGR treatment. Control cells treated with 3 μM guanosine during the first cell cycle were similarly labeled with [^{3}H]thymidine. The results of two separate experiments are shown. The curves on the left were obtained from cells treated with TGR; the curves on the right were obtained from cells treated with an equitoxic dose (35 μM) of SeGuR. As described in Results, all effects of SeGuR are probably attributable to contaminating TGR. All cells were harvested 24 hr after the first mitosis. Aliquots of [^{14}C]-and [^{3}H]-labeled cells were applied to the same filters. The results are expressed as the percentage of [^{14}C]DNA relative to [^{3}H]DNA retention. Thus, SSB are indicated by decreased retention in the curve ●——●. DPC in TG-containing DNA but not daughter DNA are suggested by the greater retention of [^{14}C]DNA given by the curve △——△. Absence of significant double strand breaks (DSB) is suggested by the curve ○——○. The total ^{14}C and ^{3}H applied to the filters were 2900 and 27,600 dpm in these experiments.

is consistent with reports that TG and mercaptopurine exhibit delayed cytotoxicity, i.e. perhaps in daughter cells or in treated cells after a critical time period [17]. Such delayed effects on progression through the cell cycle [18, 19] and chromosomal abnormalities associated with drug treatment have been reported [8]. In L1210 cells, DNA damage as determined by alkaline elution analysis is more apparent in sequentially labeled DNA than in simultaneously labeled DNA, suggesting a delayed damage in the DNA of these cells as well [4, 5]. These delayed actions of the drug imply a form of memory that ultimately leads to cell death; alternatively, persistence of the drug at critical targets in

Table 3. Inability of Chinese hamster ovary cells to remove incorporated 6-thioguanine from DNA or RNA*

Sample	TG nucleotide (dpm × $10^3/10^7$ cells)		
	Time after addition of nonradioactive TG		
	0 hr	1 hr	6 hr
Acid-soluble	24.3	10.1	6.54
RNA	16.7	15.5	14.3
DNA	14.9	14.9	16.2

* CHO cells were incubated with [^{14}C]TG (0.2 μM) for 24 hr. They were then washed, and nonradioactive TG was added. The cells were extracted with cold perchloric acid at 0, 1, and 6 hr after the addition of nonradioactive TG. The amount of radioactivity in the cold, acid-soluble pool (nucleotides), as well as RNA or DNA, was determined as previously described [17]. Similar results were obtained whether or not nonradioactive TG was added.

the treated cells may account for the delayed toxicity. TG was not readily removed from the nucleic acids of CHO cells (Table 3). This finding is consistent with that of LePage and Jones [20] in ascites tumor cells in mice. Thus, persistence of the drug in nucleic acids may be associated with the delayed cytotoxicity.

The observation that TG produces damage later after its incorporation into DNA suggests that DNA synthesis may progress normally during TG incorporation and that synthesis may be impaired in subsequent replication attempts. This idea was tested in synchronized CHO cells by evaluating DNA elongation when TG was being incorporated (simultaneous exposure) or in the cell cycle after TG treatment (Fig. 3). Since DNA elongation was impaired more markedly in the cell cycle after TG treatment, it is possible that replication of a TG-DNA template does not proceed in a normal manner. Consistent with these observations in intact cells, elongation of DNA synthesized in an *in vitro* system was reported to occur at a normal rate when deoxythioGTP was used in place of dGTP [21].

In summary, these results demonstrate in CHO cells that the DNA containing TG and, possibly, DNA synthesized therefrom, exhibit SSB. Further, the types of DNA damage observed may vary with the type of cell studied. Since incorporation into DNA has clearly been demonstrated to be associated with both DNA damage and cytotoxicity [2], elucidation of the mechanisms responsible for the damage would clarify the biochemical actions of this agent.

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