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CONCURRENT UNILATERAL CHROMATID DAMAGE AND DNA STRAND BREAKAGE IN RESPONSE TO 6-THIOGUANINE TREATMENT

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Abstract—The delayed cytotoxicity of 6-thioguanine (TG) may relate to the arrest of cells in G_2 upon completion of one cell cycle after drug exposure. In Chinese hamster ovary (CHO) cells, both the unilateral chromatid damage in G_2 chromosomes, determined by induction of premature condensed chromosome condensation [Maybaum and Mandel, *Cancer Res.* **43**, 3852 (1983)], and incorporation of TG into DNA resulting in DNA strand breakage [Christie *et al.*, *Cancer Res.* **44**, 3665 (1984)] were correlated with cytotoxicity. We have studied the correlation between strand breakage and unilateral chromatid damage in L1210 cells. DNA breaks were detected only when cells were treated with TG (0.25 μM) for one cell cycle time (12 hr) followed by 12 hr in drug-free medium containing [^{3}H]thymidine (TdR) to label the DNA. After simultaneous incubation of cells with drug and label during the first or second 12-hr period, strand breaks were not found. Strand breaks increased with dose, which correlated with greater cytotoxicity (0.01 to 0.25 μM). Treatment of cells with 0.25 μM TG for 12 hr, and transfer to drug-free medium for 12 hr prior to making prematurely condensed chromosomes (PCC), resulted in unilateral chromatid damage. Prominent curving of G_2 chromosomes with gapping and diffuse staining of one of the sister chromatids occurred. The 4-fold increase in the percentage of cells in G_2 compared with control cells suggested G_2 arrest. When cells were treated with TG for 12 hr and PCC made immediately, neither the arrest of cells in G_2 nor unilateral chromatid damage was observed. These data suggest that strand breaks and unilateral chromatid damage occur in the second cell cycle after TG exposure and that this damage may be important in TG-delayed cytotoxicity.

The antimetabolite, 6-thioguanine (TG), must be anabolized to thioguanylate to have activity as an antitumor agent [1]. TG, once activated, can alter cellular biochemistry at several sites. For example, TG depresses *de novo* purine synthesis through inhibition of purine ring formation or purine interconversion [2–4]. These effects on purine metabolism, however, have not always correlated with growth inhibition or cytotoxicity [5]. TG has also been found to replace guanine residues in both RNA and DNA [6, 7]. Although RNA synthesis may be a target for TG activity [8, 9], TG incorporation into DNA and the subsequent effects of its incorporation on DNA structure and function have been the focus of many investigations [7, 10–13]. TG is incorporated into DNA in a dose-dependent manner, and the extent of TG incorporation was correlated with cytotoxicity [7]. Although TG incorporation into DNA is probably a key event in the production of cytotoxicity, other events must occur before TG-induced toxicity is observed. Several investigators have found that mid to late S phase cells exposed to TG complete DNA synthesis and progress through G_2 into mitosis [11, 14]. After cell division, the drug-treated cells continue through a second S phase and subsequently become arrested in G_2. Thus, it appears that arrest in G_2 is associated with the delayed lethality induced by TG [11].

Antitumor drugs which cause G_2 arrest have been shown to damage chromatin [15]. TG treatment has also been shown to alter chromatin structure [12, 16] and cause DNA strands breaks [7]. The damage to G_2 chromatids as visualized by premature chromosome condensation consisted of curving and kinking of Chinese hamster ovary (CHO) cell G_2 prematurely condensed chromosomes (PCC) and unilateral gaps and diffuse staining [12, 16]. The physical damage to the chromatin was dose dependent and correlated with the delayed cytotoxicity [12]. Cytotoxicity has also been associated with TG incorporation into DNA and subsequent production of single-strand breaks in CHO cells [7].

In the studies with L1210 cells reported here, DNA breakage, as detected by alkaline elution methods, and chromatin damage, as assessed by PCC analysis, have been correlated with drug-induced cytotoxicity. These studies suggest that the unilateral chromatid damage found after TG treatment may be a more general phenomenon and not restricted to a single cell type. Furthermore, these data support the hypothesis that TG-induced toxicity requires cell progression and a second period of DNA replication after the incorporation of the drug into the DNA. This second period of DNA synthesis results in

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chromatin damage and DNA strand breakage which are correlated with cytotoxicity.

MATERIALS AND METHODS

Drugs and chemicals. TG was dissolved in double-distilled H_2O, and the concentration was determined by measuring the absorption at 321 nm. [^{3}H]Thymidine (TdR) (80 Ci/mmole) and [^{14}C]TdR (56 mCi/mmole) were obtained from RPI (Mount Prospect, IL) and Moravek Biochemicals, Inc. (Brea, CA) respectively. Ultraviolet-irradiated Sendai virus was provided by Dr. Walter Hittelman, Department of Developmental Therapeutics, the University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, TX. Colcemid, RPMI 1640, alpha-minimum essential medium (MEM), dialyzed horse serum and calf serum were obtained from GIBCO Laboratories (Grand Island, NY). Fetal calf serum was obtained from Biofluids, Inc. (Rockville, MD). Unless otherwise noted, all other chemicals were obtained from standard chemical sources and were of the highest purity available.

Cell culture. L1210 cells were maintained in continuous suspension culture in RPMI 1640 medium supplemented with 10% dialyzed horse serum, penicillin G (10^5 units/l) and streptomycin (100 mg/l). Cells were passaged twice weekly, and a doubling time of 12 hr was found. CHO cells were grown in monolayer culture in alpha-MEM with 10% calf serum and 5% fetal calf serum. The doubling time for these cells was 12 hr. Both cell lines were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°.

Colony forming assays. The viability of L1210 cells after vehicle or drug treatment was assessed using the soft agar cloning method of Chu and Fischer [17]. Cells were treated with TG for 12 hr and washed twice, then diluted to a known cell number in RPMI 1640 supplemented with 20% dialyzed horse serum and agar (0.12%). For each drug concentration, a minimum of three different dilutions of cells were plated in triplicate and incubated for 10–12 days in a humidified atmosphere of 95% air–5% CO_2 at 37°. Colonies were counted and expressed as a percentage of control non-drug-treated cells. Control colony forming efficiencies were typically between 80 and 90%.

Premature chromosome condensation. Visualization of unilateral chromatid damage and disturbances of cell cycle progression were assessed by premature chromosome condensation as described by Hittelman and Rao [18]. L1210 cells were exposed to TG for 12 hr. The drug was removed and the cells were resuspended in drug-free medium for an additional 12 hr, after which time PCC were made. Another group of cells was exposed to TG for 12 hr and PCC were made immediately. Control cells received no drug treatment. Three million control or TG-treated L1210 cells were combined with one million mitotic CHO cells in 5 mM piperazine-*N*-*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer at pH 7.4 and mixed thoroughly. Mitotic CHO cells were prepared by mitotic detachment of cells that had been treated with 0.05 μg/ml colcemid for 3 hr.

The L1210/mitotic CHO cell mixture was centrifuged and resuspended in 0.5 ml of Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, NY) containing approximately 4000 hemagglutinating units of Sendi virus per ml of HBSS and then placed in an ice water bath at 4° for 15 min. The virus–cell fusion mixture was transferred to a 37° water bath for 45 min after the addition of 50 μl of an HBSS solution containing 100 mM $MgCl_2$ and 4 μg/ml colcemid. The cells were swollen hypotonically by the addition of 7 ml of 0.075 M KCl and incubated at room temperature for 12 min. Cells were collected by centrifugation, fixed with methanol/glacial acetic acid (3:1), and then dropped onto clean, wet microscope slides. The air-dried slides were stained with 2% aceto-orcein (GIBCO). The slides were coded and then scored blindly using a light microscope. The spreads were located at 400× and then examined at 1000× to determine the PCC morphology. At least 100 PCC spreads were scored for each treatment group.

Alkaline elution assay for single-strand breaks. Alkaline elution methods were used to assess DNA strand breaks according to the method described by Kohn *et al.* [19]. L1210 cells were labeled with [^{3}H]TdR (0.1 μCi/ml, 1 μM) and treated with TG in one of four ways, as illustrated in Fig. 1, before analysis by alkaline elution methods. One group of cells (I) was simultaneously treated with [^{3}H]TdR and 0.25 μM TG for 12 hr and then incubated for an additional 12 hr in fresh medium. Group II cells were treated in a similar fashion, except that alkaline elution analysis for single-strand breakage was performed immediately after TG exposure for 12 hr. A third group of cells (III) was prelabeled with [^{3}H]TdR for 12 hr. After labeling, the medium was removed and new medium containing 0.25 μM TG was added for 12 hr. Group IV cells were exposed to 0.25 μM TG for 12 hr. After drug exposure, the medium was removed and replaced with drug-free medium containing [^{3}H]TdR. Group IV cells were incubated for an additional 12 hr prior to analysis of DNA single-strand breaks by alkaline elution methods.

Control cells were labeled with [^{3}H]TdR or [^{14}C]TdR (0.01 μCi/ml, 1 μM) for 12 hr. Control ^{3}H-labeled cells received either 0 or 300 rads of X-irradiation on ice just prior to alkaline elution analysis. Internal standard cells (labeled with [^{14}C]TdR)

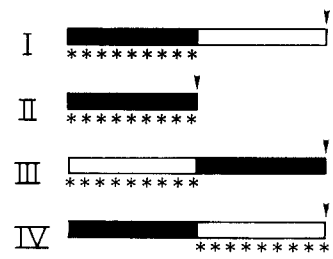

Fig. 1. Schedule of drug treatment and labeling used in alkaline elution experiments. Closed bars represent 12-hr exposure to TG-containing medium, open bars indicate 12-hr incubation in drug-free medium, asterisks indicate the presence of [^{3}H]TdR, and arrows indicate the time at which cells were harvested for elution analysis.

received 300 rads of X-irradiation on ice just prior to alkaline elution analysis. All cells were kept at 4° in the dark until analyzed. Aliquots of experimental (^{3}H-labeled) cells and the internal standard (^{14}C-labeled) cells were mixed and applied to 2 μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA). The cells were then lysed using a lysis buffer containing 5% sodium dodecyl sulfate, 20 mM EDTA and 100 mM glycine at pH 10. This solution was drained by gravity, and 2 ml of the lysis solution containing proteinase K (0.5 mg/ml) was pumped through the filter at the rate of 2 ml/hr to remove protein associated with the DNA. The DNA was eluted with 40 ml of solution containing 0.1 M tetrapropyl ammonium hydroxide, 20 mM EDTA, and 0.1% sodium dodecyl sulfate, pH 12.1, at a rate of 2 ml/hr. Fractions were collected directly into miniscintillation vials at 90-min intervals for 15 hr. Liquiscint (National Diagnostics, Somerville, NJ) counting solution containing 0.7% glacial acetic acid was added, and the fractions were counted on a Beckman LS 6800 liquid scintillation counter. The filters were collected, and the residual DNA was hydrolyzed with 0.4 ml of 1 N HCl for 1 hr at 90° to determine the amount of radioactivity remaining on the filter. Scintillation counting was carried out as described above. To remove any residual radioactivity from the pump tubing or system, 2 ml of 1 N NaOH was flushed through the system, collected as a single fraction, and counted.

To determine the total [^{3}H] and [^{14}C] contained in a sample, the amounts in the individual fractions were summed along with that contained in the lysis solution, the NaOH fraction, and the filter fraction. The fraction of [^{3}H] (experimental cells) remaining on the filter was plotted against the fraction of [^{14}C] (internal standard cells) remaining on the filter.

RESULTS

Cytotoxicity studies. L1210 cells exposed to increasing concentrations of TG for 12 hr showed a dose-related decrease in survival as assessed by colony formation in soft agar (Fig. 2). The threshold for toxicity was 0.01 μM. At a concentration of 0.25 μM TG, a 2 log cell kill was found. Morphologic and biochemical analyses of TG-induced damage were conducted in cells exposed to 0.25 μM drug.

Cell cycle position classified by PCC morphology. L1210 cells were treated with TG or vehicle and fused with mitotic CHO cells to make PCC as described in Materials and Methods. PCC spreads were scored as to their position in the cell cycle, based on chromatin morphology. The classification scheme relies on the observation that, as cells progress from G_1 into S, the chromatin decondenses in preparation for DNA synthesis in a characteristic manner [18]. Decondensation is manifested in two ways. First, chromatin structures elongate. Second, discontinuities are observed as apparent breaks or gaps in the chromatin. These discontinuities are not frank breaks in DNA structure, but represent areas in which DNA has become unwound to such an extent that it is no longer visible under the light microscope. As cells progress from S into G_2, bifilial PCC are apparent [18, 20].

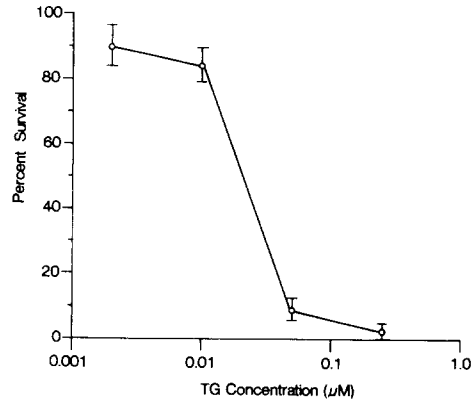

Fig. 2. L1210 cell survival after 12-hr exposure to TG. L1210 cells in logarithmic growth were exposed to TG (0.0025 to 0.25 μM) for 12 hr and then prepared for colony forming assays as described in Materials and Methods. Data are expressed as a percentage of untreated control cells. Each point represents the mean ± S.E. for at least three determinations.

The classification scheme consisted of four PCC groups in addition to the distinct bifilial G_2 PCC. Group 1 consists of PCC that are highly to moderately condensed single chromatid structures with few or no discontinuities and which arise from cells in early to mid G_1 (see Fig. 3). In group 2 spreads, the chromatin is more extended than in group 1 and some areas of discontinuity are found. The chromatin is mostly discontinuous in group 3, although some regions of extended chromatin still exist. Spreads classed as 2 and 3 arise from cells that are in late G_1 or early S. In group 4, the chromatin is maximally decondensed and completely discontinuous in appearance and arises from cells in S phase. PCC spreads which were completely or nearly completely bifilial were classified as G_2. Spreads having this morphology come from cells that have finished replication and have initiated recondensation in preparation for mitosis [20]. These classifications are based upon the classical descriptions reported by Rao and Johnson [20] and further discussed by Rao *et al.* [21]. Although no other methods were used in the present study to correlate PCC morphology and cell cycle position, prior work in this field has demonstrated the usefulness of this technique in assessing cell cycle position after drug treatment [12, 18, 20].

Classification of unilateral chromatid damage in vehicle or drug-treated cells. The distinctly bifilial G_2 PCC spreads were further classified as to the degree of unilateral chromatid damage. This unique type of damage to chromatid structures after exposure to TG was described originally in G_2 PCC of CHO cells [16]. This form of chromosomal disruption is distinct from the usual gaps, breaks and exchanges often observed with other DNA damaging agents [22, 23]. Unilateral chromatid damage was observed as curling and kinking of G_2 PCC. In addition, numerous regions which were diffusely stained or gapped were found in the kinked and curled chromatid. The sister chromatid, however, showed no evidence of damage. In the studies reported here in L1210 cells, we also

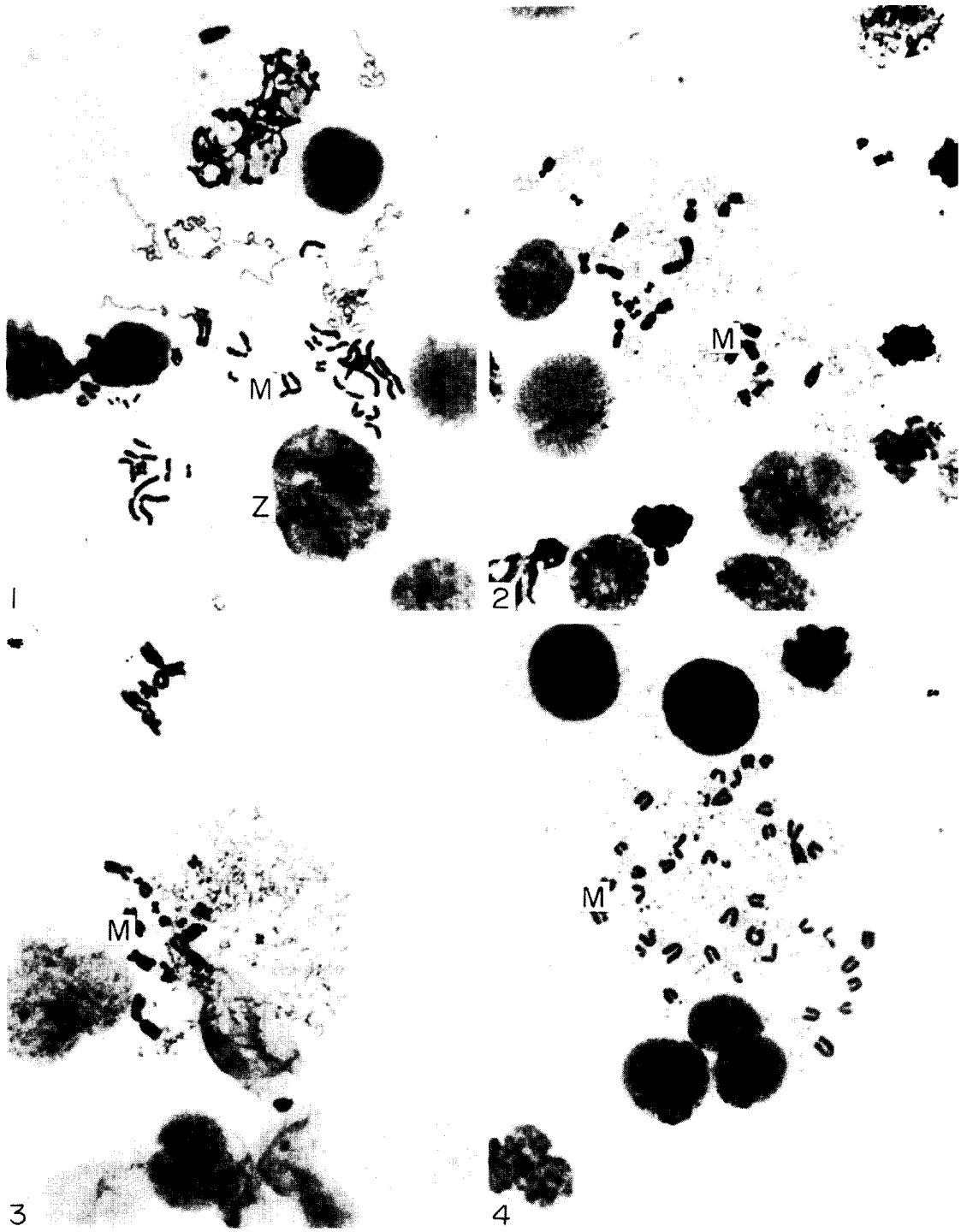

Fig. 3. Photomicrographs of PCC decondensation groups of L1210 cells. The spreads pictured here are representative of the degree of chromatin decondensation, which was the criterion used to assign a PCC spread to either group 1, 2, 3, or 4. The characteristics of each group are discussed in Results. "M" indicates CHO cell mitotic chromosomes. An example of G_2 PCC spreads is found in Fig. 4.

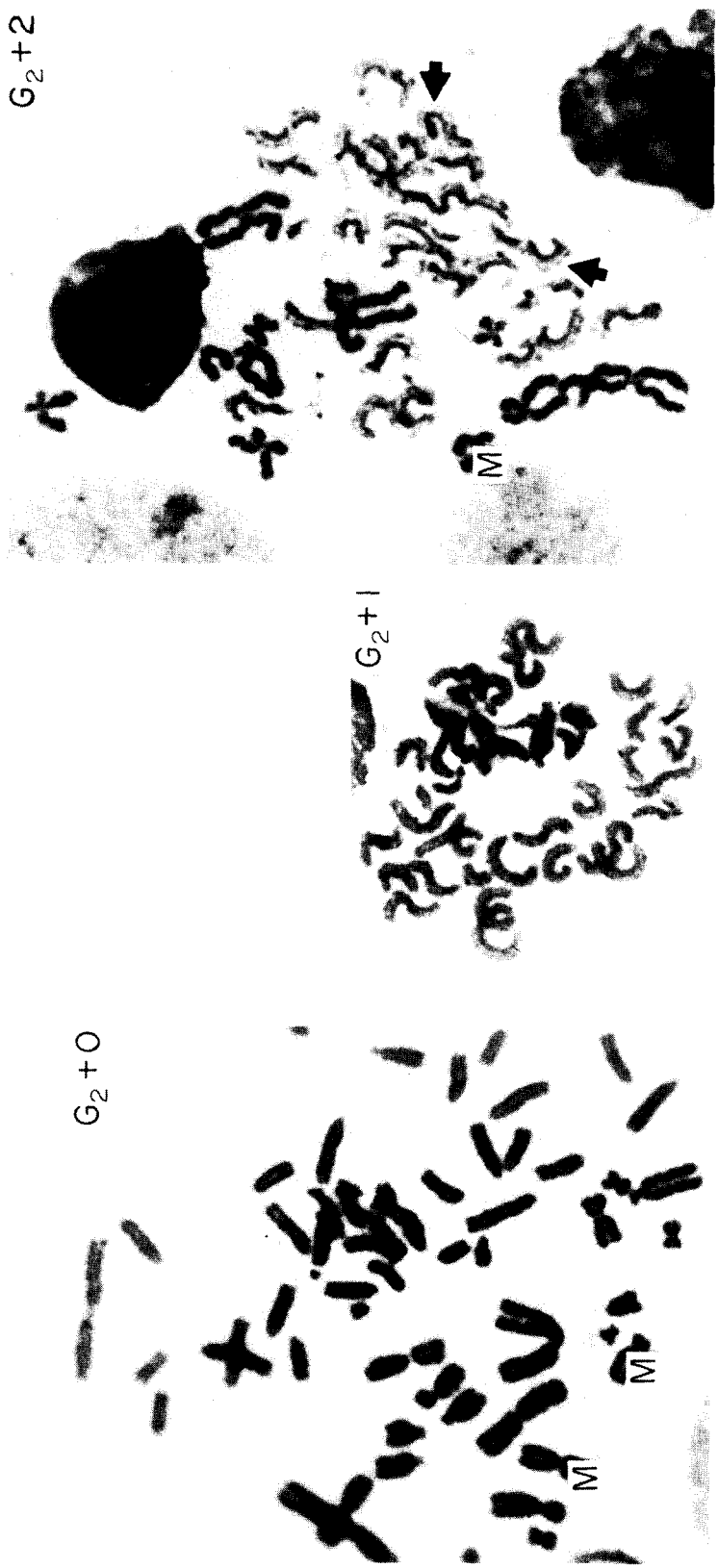

Fig. 4. Damage to G_2 PCC resulting from TG treatment. L1210 cells were treated with TG (0.25 μM) and then PCC were made as described in Materials and Methods. The spreads shown in the figure indicate the types of damage which were found in G_2 PCC. Spread +0 is representative of spreads which were classified as normal or nearly normal, while +2 shows the most severe disruption of G_2 PCC. Arrows indicate unilateral chromatid damage and "M" indentifies mitotic CHO cell chromosomes.

found unilateral chromatid damage in G_2 cells. This damage was classified according to the degree of deformation observed and assigned to one of three categories, G_2-+0, G_2-+1, or G_2-+2 (Fig. 4). Spreads that contained less than three kinks were assigned a deformation score of G_2-+0. In this scoring system, a "kink" was defined as a bend in the G_2 chromatid of at least 90° which occurred over a distance of not more than twice the width of the PCC. A curved region of the chromatid that included the centromere was not classed as a kink. To be assigned a G_2-+1 deformation score, a spread had to contain PCC with at least three kinks, but no differential chromatid staining. When at least three kinks and regions of differential chromatid containing were present, the spread was assigned a deformation score of G_2-+2. If it was unclear as to which class a particular spread should be assigned, the lower of the two classifications was used. All slides were coded before the slides were scored for unilateral chromatid damage by an independent observer. After evaluation, the codes were broken and the accumulated data were assessed. In no manner does the scoring system used to classify the unilateral chromatid damage found in G_2 PCC imply any progression through the G_2 phase of the cell cycle.

Analysis of TG-treated cells by PCC decondensation morphology. To analyze cell cycle position after TG treatment, the decondensation morphologies of PCC were classified. Figure 5 shows PCC decondensation histograms for three types of drug treatment: vehicle-treated control cells (treatment A), cells treated with TG (0.25 μM) for 12 hr and then analyzed by premature chromosome condensation (treatment B), or cells treated with TG (0.25 μM) for 12 hr followed by an additional 12 hr in drug-free medium with subsequent PCC analysis (treatment C). Treatment A shows a typical distribution of PCC spreads for vehicle-treated control cells. As described above (Fig. 3), an increase in the numerical value of the decondensation score represents progression through the cell cycle from G_1 into S, with group 4 representing S-like PCC. For vehicle-treated cells, 80% of the total spreads scored were found in groups 2, 3 or 4. G_2 spreads made up 15% of the total number scored. With treatment B (12-hr TG treatment and then immediate PCC analysis), the distribution of spreads was similar to that observed in control cells. Although about half as many group 3 spreads were found in treatment B compared to treatment A, approximately 80% of the spreads were classified as belonging in groups 2–4 and spreads in G_2 made up about 15% of the scored spreads. In contrast, treatment C (12-hr TG exposure, followed by 12 hr in TG-free medium, then PCC analysis) produced a PCC distribution that was very different from A or B. G_2 spreads constituted 67% of the total spreads scored with a corresponding decrease in the number of spreads in groups 1 through 4 compared with control. The group 4 spreads represent those cells in S phase. The percent of total spreads classified as in group 4 were equivalent for treatments A and B (22%). In contrast, for treatment C, 8% of the total spreads were classified as group 4 spreads.

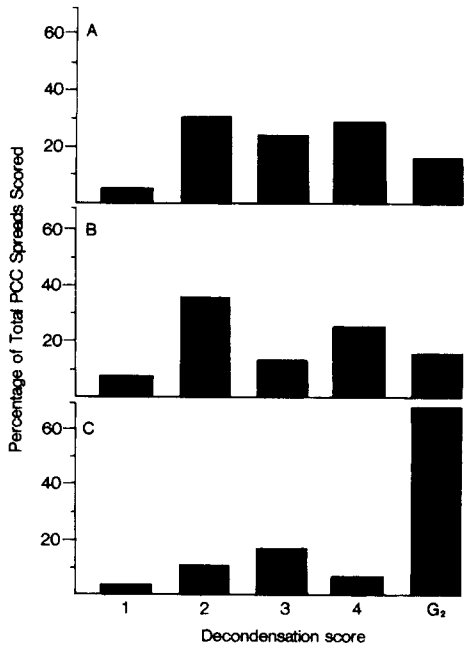

Fig. 5. Frequency distributions of PCC decondensation morphologies according to TG treatment schedule. Data are: treatment A, control cells; treatment B, exposure to TG (0.25 μM) for 12 hr followed by PCC analysis; treatment C, same as B, but incubated for an additional 12 hr in drug-free medium before PCC analysis. The results are expressed as a percentage of the total PCC spreads scored in each treatment group; 100–200 spreads were scored for each group.

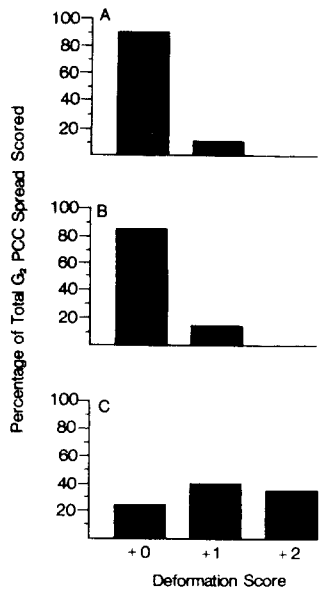

Fig. 6. Extent of G_2 PCC damage according to TG treatment schedule. Treatments A, B, and C were carried out as described in Fig. 5. The results are expressed as a percentage of the total number of G_2 PCC spreads scored for each treatment group; 30–65 G_2 PCC spreads were examined for each group.

Analysis of unilateral chromatid damage induced by TG. In addition, G_2 spreads were further classified as to the extent of unilateral chromatid damage observed, as described above, and shown in Fig. 3. Ninety percent of the G_2 PCC spreads from control cells appeared normal with virtually no kinking and were assigned a deformation score of +0 (treatment A in Fig. 6). A baseline level of moderate chromatid deformation (+1) was observed in 10% of the control G_2 spreads. Treatment B did not alter the distribution of unilateral chromatid damage in the G_2 PCC. Treatment C, however, produced a major shift in the percentage of spreads with a higher deformation score. Nearly 80% of the G_2 spreads scored had moderate to major unilateral chromatid damage with approximately equal numbers of G_2 spreads in each category (+1 or +2 deformation scores). Only 20% of the G_2 spreads analyzed in treatment C were found to exhibit normal G_2 PCC morphology (+0 deformation score) with respect to kinking or differential chromatid staining.

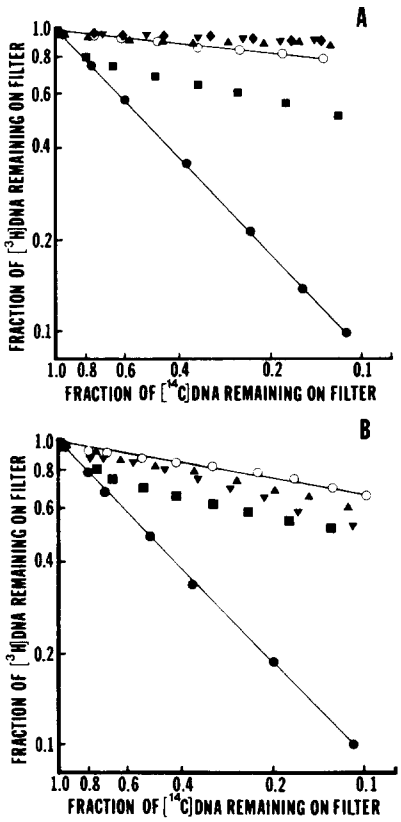

Fig. 7. DNA single-strand break production in L1210 cells according to TG schedule (A) and dose (B). Panel A, treatment schedules (see Fig. 1) were: I (▲) simultaneous ^{3}H-labeling and TG exposure for 12 hr followed by 12 hr in fresh medium; II (♦) simultaneous ^{3}H-labeling and TG exposure for 12 hr; III (▼) ^{3}H-prelabel for 12 hr followed by 12-hr TG exposure without label; IV (■) TG exposure for 12 hr followed by 12 hr ^{3}H-label. All TG treatments were with 0.25 μM drug. In Panel B, L1210 cells were treated with different doses of TG according to schedule IV. TG doses were: 0.01 μM (▲), 0.025 μM (▼), and 0.25 μM (■). In both panels (○) represents control cells without treatment and (●) represents cells which received 300 rads of X-iradiation before alkaline elution. In all cases, ^{14}C-labeled cells were given 300 rads of X-irradiation prior to alkaline elution.

Assessment of TG-induced strand breaks by alkaline elution. Alkaline elution was used to determine the ability of TG to cause breaks in the DNA of L1210 cells. Strand breaks were monitored after four different schedules of TdR labeling of DNA and TG treatment as described in Materials and Methods and illustrated in Fig. 1. Figure 7A shows that group IV produced the most rapid rate of elution, indicating that this labeling and treatment schedule produced DNA strand breaks. The single-strand break frequency in rad-equivalents for the experiment in Fig. 7A was 113. Cells treated according to schedules I, II, or III eluted from the filters at rates similar to that observed in non-drug-treated cells.

Because it appeared that TG treatment followed by 12 hr post drug exposure labeling was the only schedule to result in strand breaks, a series of studies using this protocol was carried out to see if the strand break production was dose related. The results are shown in Fig. 7B. TG concentrations of 0.01, 0.025 and 0.25 μM gave increasing amounts of strand break production, in which the single-strand break frequencies in rad-equivalents for the experiment shown in Fig. 7B were 34, 51, or 101 respectively.

DISCUSSION

Both unilateral chromatid damage [12, 16] and DNA strand breakage [7, 13] have been proposed as mechanisms for cytotoxicity induced by TG. Other investigations have suggested that TG toxicity is delayed [11, 12, 14, 16] with the appearance of chromatid damage in the cell cycle following TG incorporation [11, 12, 16]. Correlation of DNA strand breakage and/or chromatid damage with cytotoxicity has been difficult due to the use of different cell lines. The focus of this study was to correlate the production of unilateral chromatid damage and DNA strand breakage by TG in L1210 cells, which are sensitive to TG-induced toxicity. In addition, the studies reported here extend previous investigations which attempted to explain the mechanisms for delayed cytotoxicity of TG. L1210 cells incubated with 0.01 to 0.25 μM TG for 12 hr showed a dose-related increase in cytotoxicity. At the lower dose (0.002 μM), no apparent cytotoxicity was found. The concentration of TG required to produce a 50% reduction in survival after a 12-hr exposure was estimated to be 0.02 μM. Increasing the exposure time for TG to 16 hr lowered the LC_{50} to 0.008 μM (data not shown). In CHO cells exposed to TG for 16 hr, 0.5 μM drug produces a 50% decrease in survival [12]. When a CHO cell subline, AA8, which was competent in DNA repair was studied, the reported LC_{50} was approximately 1 μM after 24 hr of exposure [7]. These results suggest that L1210 cells are 25- to 125-fold more sensitive to TG than CHO cells.

Because L1210 cells are more sensitive to TG, unilateral chromatid damage and DNA damage should occur at lower concentrations of TG, if these manifestations of nuclear disruption play an important role in TG lethality. Maybaum and Mandel

[12, 16] demonstrated chromatid disruption in CHO cells after the cells were exposed to TG. Two forms of chromatid disruption were observed by these investigators. At lower doses, 80% of the G_2 PCC spreads exhibited prominent kinking [12, 16]. At a higher dose (4 μM), unilateral chromatid damage in the form of diffuse staining and gapping occurred in 20% of the G_2 PCC spreads in addition to prominent kinking (80% of total G_2 spreads) [12, 16]. Furthermore, kinking was always observed in spreads which exhibited unilateral chromatid damage [12, 16]. In the studies reported here, L1210 cells treated with 0.25 μM TG exhibited extensive chromatin damage. Unilateral chromatid damage was found in 40% of the G_2 PCC spreads (Fig. 6). The damage observed in L1210 cells (Fig. 6) was very similar to that seen in CHO cells [12, 16]: kinking in moderately damaged chromatin, while more severe disruption was manifest as kinking and unilateral chromatid damage. These data suggest that TG-induced toxicity is associated with the presence of unilateral chromatid damage and/or kinking in cell lines sensitive to TG. Furthermore, the unilateral chromatid damage induced by TG is not unique to CHO cells but may be a general feature of delayed TG toxicity.

TG is known to incorporate into DNA, but this incorporation does not affect DNA synthesis immediately because TG-treated cells are able to complete S phase and divide [11, 14]. It is only after a second round of DNA synthesis that cell progression is disrupted, and the cells become arrested in G_2 [11]. The irreversible arrest in G_2 of TG-treated cells has been associated with a primary event leading to cell death. Data presented here and by Maybaum and Mandel [12, 16] suggest that the G_2 arrest of TG-treated cells may result from the disruption of chromatin structure which was manifested in these studies by kinking and unilateral chromatid damage. When L1210 cells were exposed to 0.25 μM TG for 12 hr and then PCC were made immediately (treatment B), no disruption of G_2 PCC was seen (Fig. 6), nor was there arrest of cells in G_2 (Fig. 5). When the cells were treated with TG for 12 hr followed by a 12-hr period in drug-free medium (treatment C), cells were arrested in G_2 (Fig. 5) and chromatin disruption was observed in G_2 PCC (Fig. 6). The doubling time for the L1210 cells employed in the present report was 12 hr. Taken together the data suggest that at least two rounds of DNA synthesis must occur in order for TG-induced arrest in G_2 and chromatin damage to be manifested in L1210 cells. In the present studies, the schedule which produced both G_2 arrest and chromatin damage was treatment C. Treatment C permitted TG to be incorporated into DNA during the first 12 hr of incubation, corresponding to one doubling time for L1210 cells. Other investigators have demonstrated that the incorporation of 2′-deoxy-6-thioguanosine-5′-triphosphate into DNA proceeds efficiently when normal DNA is used as a template [24]. This first round of replication in L1210 cells would lead to the presence of TG in one strand of the DNA. After 12 hr in drug-containing medium, the cells were washed and placed in fresh medium for an additional 12 hr to permit a second round of DNA synthesis in the absence of TG. It is possible that, during this second round of DNA synthesis, the TG-containing DNA strand acts as a faulty template and results in the production of kinked and/or unilaterally damaged chromatids. It is not known whether TG-containing DNA can be replicated in the normal manner. It has been shown, however, that the transcription of synthetic DNA substituted with TG is inhibited [25].

Chromatin damage induced by TG appears to occur in the newly synthesized DNA that does not necessarily contain TG, but which is replicated from the TG-containing DNA strand. This is demonstrated by the appearance of strand breaks only when DNA is labeled during the second round of replication after TG treatment (Fig. 7A, group IV). This would suggest that TG is incorporated into DNA in a normal fashion, but the TG-containing template causes the subsequent synthesis of damaged DNA leading to the appearance of unilateral chromatid damage. Other investigators [7, 13], in contrast, have reported the presence of DNA strand breaks occurring early after TG treatment, suggesting that the damage could occur in the parental strand at high drug doses. It is possible that this discrepancy is due to different drug doses employed in the various studies.

Maybaum and Mandel [12] suggest that chromatin disruption which occurs in TG-treated CHO cells represents a change in chromosome structure or composition. Furthermore, they suggest that the distortion observed may be due to either breaks in the DNA occurring in the affected region or the failure of the chromatin to condense properly [12]. The data presented here support the former hypothesis. DNA strand breaks, as measured by alkaline elution methods, were observed only when [^{3}H]TdR was incubated for 12 hr with cells that had been pretreated with TG. Coincubation of TG and [^{3}H]TdR during the initial 12-hr period produced no discernible DNA strand breaks. These data suggest that TG incorporation into DNA alone does not produce measurable strand breaks. It is only when [^{3}H]TdR labeling occurs of cells whose DNA is already substituted with TG that the strand breaks are apparent. Other investigators have found DNA strand breaks in TG-treated cells but did not report the time schedule dependence for effects of drug and TdR labeling schedules in their studies [7]. The appearance of DNA strand breakage and unilateral chromatid damage during the same time sequence suggests that these two manifestations of TG-induced damage may play a critical role in TG lethality. Further work will be required to determine if the correlations among DNA damage, DNA strand breakage and cytotoxicity that we have observed are merely the result of drug treatment or the cause of cell death *per se*.

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