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REDUCED THIOL CONTENT IN L1210 CELLS TREATED WITH BSO INCREASES DNA CROSSLINKING BY MELPHALAN

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SUMMARY: Endogenous thiols such as glutathione (GSH) are known to mediate the activity of bifunctional alkylating agents such as melphalan (L-PAM). GSH levels can be reduced by cell growth in the presence of an analog of gamma glutamyl, α-aminobutyrate, L-buthionine (S/R) sulfoximine (L-BSO). L-1210 murine lymphocytic leukemia cells grown in vitro in the presence of 10 μM < 15% L-BSO possessed 1% of the normal GSH levels. This pretreatment regimen did not significantly alter cell viability but did enhance the cytotoxicity produced by a 15 μM-1hr L-PAM treatment. The increased cell killing correlated with enhanced DNA-DNA crosslinking immediately following L-PAM exposure. No effect of BSO pretreatments on the incomplete removal of crosslinks over 36 hr of observation was seen. These results suggest that GSH levels modulate the initial degree of L-PAM-induced DNA crosslinking, but not the long term repair of these lesions.

INTRODUCTION: Melphalan (1-phenylalanine mustard or L-PAM) is an antineoplastic agent used in a variety of hematologic and solid tumors [1]. The mechanism of action of L-PAM is believed to involve the formation of interstrand crosslinks in DNA [2,3]. These DNA-DNA crosslinks have been shown to slowly form over several hours following a brief drug exposure [3] and, in contrast to alkylating agents such as mechlorethamine (nitrogen mustard), L-PAM-induced crosslinks are removed very slowly over time [3]. Previous studies have shown that L-1210 mouse leukemia cells which develop resistance to L-PAM have enhanced levels of reduced glutathione [4,5]. Glutathione levels can be decreased by inhibition of gamma glutamyl-cysteine-synthetase using the substrate antimetabolite, L-buthionine (S/R) sulfoximine, both in vitro [5] and in vivo [6]. As a result of this inhibition, L-PAM sensitivity can be restored to resistant tumor cells (4,5). In the current communication, the effect of BSO pretreatment on the

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extent and time-course of DNA crosslinking by L-PAM was investigated in normal L-1210 cells.

METHODS

Reagents. L-1210 cells were maintained in RPMI 1640 medium fully supplemented with glutamine and 10% fetal calf serum (Flow Laboratories, Rockville, MD). L-PAM was obtained in a powdered form from Sigma Chemical, St. Louis, MO. For use in the cell cultures, the powder was initially diluted into acidified alcohol which was then diluted into phosphate buffered saline, pH 7.4. L-buthionine (S/R) sulfoximine was obtained from Chemical Dynamics Corp, Plainfield, NJ. For use in the cell cultures, both the L-PAM and L-BSO were filtered through 0.22 micron filters (Millipore) prior to use.

Measurement of Glutathione. The intracellular content of non-protein sulfhydryls was estimated using Ellman's reagent (5,5'dithiobis-(2-nitrobenzoic acid), obtained from Sigma Chemical. For each assay, 10^6 logarithmically growing L-1210 cells were lysed in distilled water and proteins were precipitated using 5% sulfosalicylic acid. Care was taken to examine cell populations at equivalent days (3) after subculturing to reduce the effect of serial decrements in GSH content with time after passage [7]. The lysate is then reacted at room temperature with reagent according to published procedures [8]. Absorbance due to non-protein sulfhydryls was then measured spectrophotometrically at 412 nm.

Experimental Treatments. Log phase cells were grown for 36 hours in complete medium containing (2- ^{14}C) thymidine (0.1 uCi/ml, 55 mCi/mmole from Research Products International Corp., Mount Prospect, IL). Thiol levels in cells were depleted by growth in medium containing ^{14}C-thymidine as above and 10 uM L-BSO. BSO was also maintained in a non-radioactive "chase" medium but was not present during or after L-PAM treatments. All L-PAM exposures were performed for 1 hr at 37^{o}C in the dark after which the cells were washed twice into fresh medium by centrifugation (X 800 g for 10 min). The time course of L-PAM effects on DNA was studied by assaying for DNA-DNA crosslinks after various periods of incubation at 37^{o}C following the 1 hr 1-PAM exposure. Viability was assessed by colony forming ability in solf agar [9]. Colonies of $>$ 60 micron size were enumerated using an automated imageanalysis system (FAS-II Omnicon TM, Bausch and Lomb, rochester, NY [10]). A 1 hr L-PAM concentration of 15 uM was selected for these studies since this is within the reported LD_{30-50} for this cell line, and because this concentration has previously been used in L-PAM mechanistic studies using alkaline elution techniques [3]. The concentration of L-BSO was 10 uM which was maintained for 48 hours. This concentration was previously shown to deplete GSH levels in L-1210 cells without appreciably reducing cell viability [6].

Alkaline DNA Elutions. The specific procedure for the alkaline elutions involved radiation of cells to 6.5 cGy on ice using a linear accelerator (Varian Associates, Palo Alto, CA). This produces a proportional frequency of strand breaks which causes the DNA to rapidly elute in alkaline conditions unless crosslinking is present [11]. Cells were then loaded at a concentration of 10^6 onto 25 mm, 2 uM polyvinyl chloride filters (Type BS, Millipore Corp., San Francisco, CA). The cells were lysed on the filters with 5 ml of pH 10.0 detergent solution containing 0.5 mg/ml of proteinase K (E. Merck Co., Darmstadt, GER). This solution was pumped through the filters at a rate of 2.5 ml/hr to eliminate any DNA-protein crosslinks. Afterwards a solution of pH 12.1 was used to elute the DNA from the filters at a rate of 2.5 ml/hr. Fifteen hourly fraction were obtained from each filter elution and the DNA was quantitated by

scintillation counting in three volumes of fluid. Crosslinks were estimated according to the formula: Crosslink/break parameter $\sqrt{(1-Ro)/(1-R)} - 1$, wherein Ro is the fraction of DNA retained on the filters from control cells, and R is the fraction retained from treated cells [11].

RESULTS AND DISCUSSION

Both BSO and L-PAM reduced the intracellular NPSH levels in L-1210 cells in vitro (Table 1). The 35% reduction in the NPSH level with L-PAM was not significantly different from controls. In contrast, the 48 hr pretreatments with 10 μm L-BSO reduced NPSH levels to $<$ 15% of control values. This treatment had only a slight effect on L-1210 colony formation in soft agar. Conversely, the L-PAM treatment was associated with significant cytotoxicity which was moderately enhanced by the L-BSO pretreatments (Table 1). This degree of enhancement (from 35% survival to 7.5% with BSO) was significantly greater than that predicted by a simple additivity model for independently-acting cytotoxic agents [12].

When the cells were analyzed for DNA-DNA crosslinks using the alkaline elution technique, an interesting pattern emerged. As anticipated from prior studies of L-PAM-induced crosslinking [3], the degree of crosslinking

TABLE 1

EFFECTS OF L-BSO ON NON-PROTEIN SULFHYDRYL (NPSH) LEVELS AND VIABILITY OF L-1210 CELLS *

L-BSO (μm x 48 hr)	L-PAM (μm x 1 hr)	NPSH (nmole/10^6 cells)	Survival of colony-forming units (% control)
0	0	1.9 $\pm$ 0.5	100 $\pm$ 8
0	15.0	1.1 $\pm$ 0.6	35 $\pm$ 9**
10.0	0	0.3 $\pm$ 0.5**	82 $\pm$ 12
10.0	15.0	0.3 $\pm$ 0.2	7.5 $\pm$ 7#

* Mean $\pm$ S.D.; n = 4
** $p < .05$ by paired T-test compared to control
# $p < .05$ by paired T-test compared to L-PAM control

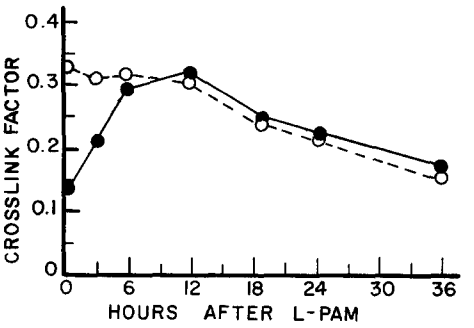

Figure 1. The effect of L-BSO pretreatment on DNA-DNA crosslinking from L-PAM in L-1210 leukemia cells. Crosslink factors (y-axis) were calculated using the formula of Kohn et al (11). Each point represents the mean of three separate experiments. Solid symbols and lines represent non-BSO treated cells; open symbols and dashed lines represent cells pretreated with 10 μM L-BSO to reduce GSH content.

increased over time reaching a peak approximately 12 hrs after a brief 1 hr exposure to the drug (Figure 1). These crosslinks were slowly and incompletely removed over the ensuing 36 hrs of observation. Figure 1 also shows the time course of L-PAM-induced crosslink formation and removal in L-1210 cells pretreated with L-BSO to reduce GSH levels. It is apparent from these results that there is a major difference in the degree of DNA crosslinking immediately following exposure to L-PAM in the GSH-depleted cells. The time course of crosslink removal was also slow as previously described (3). This process has previously been associated with the repair of alkylated DNA [3,13,14].

Another important observation in the current work was that BSO did not cause detectable DNA damage using the alkaline elution technique. This is shown in a sample elution profile wherein DNA from BSO-treated cells (both radiated and non-radiated) does not elute more slowly or rapidly than the equivalent controls (Figure 2). In this regard, BSO markedly contrasts with more DNA-reactive agents such as diethylmaleate and nitroimidazoles which have been used to deplete GSH in previous studies [15,16].

In the BSO-pretreated cells, L-PAM damage was maximal immediately following drug exposure, in contrast to the slow accumulation of crosslinks in normal L-1210 cells. The slow formation of crosslinks has been

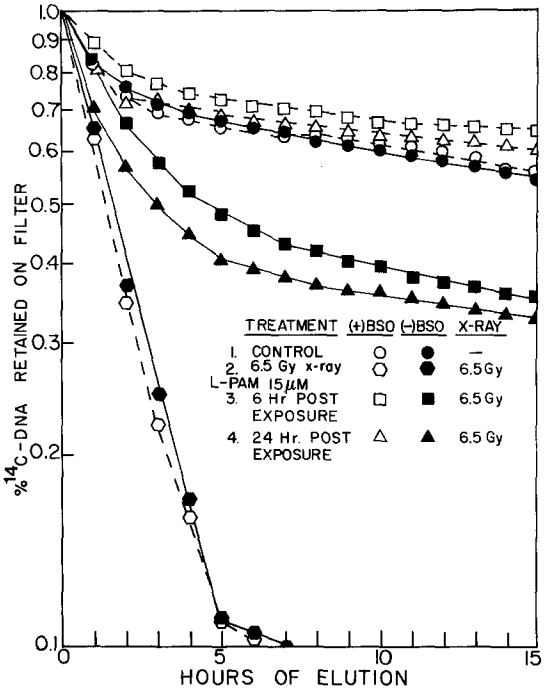

Figure 2. A sample alkaline elution profile for L-1210 leukemia cells treated with 15 μM L-PAM. Control cells were irradiated with 6.5 cGy of x-rays to induce random DNA strand breaks. The solid symbols represent cells pretreated with 10 μM L-BSO to reduce intracellular GSH content. The similarity in the elution profiles for 6 hr and 24 hr treatments reflects the slow and incomplete repair of DNA-DNA crosslinks with, or without prior L-BSO treatments. The higher retention of DNA from BSO-treated cells (open symbols) indicates a greater degree of DNA alkylation in cells depleted of GSH by L-BSO pretreatment.

previously reported for L-PAM [3] and cisplatin [17]. It is believed to involve slow chemical activation of these agents to electrophilic or coordinate-binding intermediates, respectively. The current results suggest that this activation step for L-PAM may be enhanced in GSH-depleted cells. Taylor et al have similarly demonstrated augmented rapid binding of L-PAM to cellular macromolecules [18]. This was observed in CHO cells depleted of GSH by either diethylmaleate or misonidazole.

The current results do not allow as firm a conclusion to be made about the repair of L-PAM induced DNA damage in BSO-treated cells because the rate of crosslink removal was both slow and incomplete in both cell populations. Notwithstanding this limitation, there was no difference in the rate of crosslink reversal in the presence of depressed NPSH levels. A similar finding of equivalent repair rates for L-PAM crosslinks in cells

depleted of GSH by misonidazole has been reported by a number of groups [19.20]. In one in vivo study, misonidazole pretreatments significantly lowered GSH levels in tumors and normal tissues, but this did not retard the removal of crosslinks in DNA [19]. Nonetheless, increased crosslink formation was noted commensurate with the development of depressed GSH levels in these tissues. This same pattern was observed in the current study.

Overall, these results suggest a positive correlation between enhanced cytotoxicity and rapid DNA-DNA crosslinking by L-PAM in L-1210 cells which have depressed GSH levels. The enhancement of crosslinking was immediate and may represent augmented drug activation to reactive alkylating species which are more available for covalent interactions with nuclear DNA. The slow and incomplete removal of crosslinks was not shown to be modulated by GSH levels in these cells.

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