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A COMPARATIVE ANALYSIS OF DRUG-INDUCED DNA EFFECTS IN A NITROGEN MUSTARD RESISTANT CELL LINE EXPRESSING SENSITIVITY TO NITROSOUREAS

STEPHEN W. DEAN*, ALFRED B. JOHNSON and KENNETH D. TEW†
Laboratory of Molecular Pharmacology, Division of Medical Oncology, Lombardi Cancer Research Center, Georgetown University, Washington, DC 20007, U.S.A.

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Abstract—In the Walker 256 rat mammary carcinoma cell line, WR, resistance to nitrogen mustards (NM) is accompanied by collateral sensitivity to chloroethylnitrosoureas (CENUs). DNA-interstrand cross-links, DNA-protein cross-links, and sister chromatid exchange (SCE) induction were assayed in WR and the parent cell line (WS) after treatment with nitrogen mustard (HN2), phosphoramide mustard (PM), chlorozotocin (CLZ) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU). Treatment of cells with HN2 caused extensive levels of cross-links, approximately 50% of which were DNA-interstrand, equal in both WR and WS, whereas PM caused no detectable cross-links in either cell line. CLZ induced low levels of DNA-interstrand cross-links, similar in WR and WS, but no DNA-interstrand cross-links could be detected in either cell line after treatment with CCNU. Both CLZ and CCNU induced low levels of DNA-protein cross-links in both cell lines, though higher in WR than WS. There was no difference in the rate of removal of HN2-induced DNA-interstrand or DNA-protein cross-links or total CLZ-induced cross-links by the two cell lines, suggesting that differential repair was not relevant to the expression of resistance. Both HN2 and PM caused more SCEs in WS than in WR, whereas CLZ and CCNU induced more SCEs in WR. Thus, NM-induced SCEs were related to cell killing but not cross-linking, whilst CENU-induced SCEs were related to cell killing and DNA-protein but not DNA-interstrand cross-links. Furthermore, the collateral sensitivity of WR cells to CENUs was not due to the differential induction of DNA-interstrand cross-links or repair of total cross-links, although higher levels of DNA-protein cross-links occurred in WR, and these may be either a cause or a consequence of increased susceptibility of these cells to CENUs. Presumably NMs and CENUs have several distinct and separate macromolecular targets which result in differential cell killing. It is concluded that a range of lesions occurred after treatment of WR and WS cells with either NMs or CENUs and that, in these cell lines, there is no simple correlation between drug-induced cross-linking, SCE induction and cytotoxicity.

Previous studies of nitrogen mustard (NM) and chloroethylnitrosourea (CENU) resistance in tumor cells have shown increased efficiency of DNA adduct removal in the resistant populations [1–4]. In the Walker 256 rat mammary carcinoma cell line, WR, resistance to bifunctional NMs is accompanied by collateral sensitivity to carbamoylating nitrosoureas [5]. The ability of the latter class of drugs to overcome resistance to NMs has been attributed to inhibition of glutathione reductase and concomitant depletion of intracellular thiols [6]. The increased toxicity results from the intrinsically lower levels of glutathione reductase in the WR compared to the parent cell line, WS.

The mechanism by which WR cells express cellular resistance to NMs is less well documented. A recent study revealed higher glutathione-*S*-transferase enzyme activity in the WR cell line and demonstrated a qualitative correlation between increased resistance and elevated glutathione-*S*-transferase activity [7]. The ability of these enzymes to detoxify alkylating species may, in conjunction with other reported genotypic and phenotypic alterations, assist in the expression of resistance [8, 9]. However, the precise nature of the comparative chromatin effects of nuclear reacting drugs in both WR and WS has not been characterized.

In this study, we treated WR and WS cells with two representative NMs [nitrogen mustard (HN2) and phosphoramide mustard (PM)] and CENUs [1-(2-chloroethyl)-3-cyclohexyl-l-nitrosourea (CCNU) and 2[3-(2-chloroethyl)-3-nitrosoureido]-2-deoxy-D-glucopyranose, chlorozotocin (CLZ)] and assayed DNA-interstrand crosslinks, DNA-protein cross-links, and repair of induced cross-links. We also examined the induction of sister chromatid exchanges (SCEs) by all four drugs, a phenomenon reported to reflect some aspects of DNA damage [10]. The relationship between these various endpoints should provide a better understanding of the mechanisms by which CENUs and NMs cause cell death, and the nature of the comparative resistance/sensitivity properties of the Walker cell lines.

MATERIALS AND METHODS

Cell cultures. Derivation of Walker 256 rat mammary carcinoma cells resistant (WR) and sensitive (WS) to chlorambucil has been described previously [5]. Both cell lines were maintained as static sus-

* Address all correspondence to: Stephen W. Dean, M.R.C., Cell Mutation Unit, University of Sussex, Falmer, Brighton, U.K.
† Present address: Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111.

pension cultures in Dulbecco's minimal essential medium containing 4.5 g/l glucose (Biotech. Research Laboratories, Rockville, MD) 4 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (MA Bioproducts, Walkersville, MD), supplemented with 10% fetal calf serum (FCS) (KC Biological, Lenexa, KS) under 5% CO_2. Resistance of WR is maintained by biannual exposure to 20 μg/ml chlorambucil, followed by serial passage as usual.

Drugs. CCNU, CLZ, HN2 and PM were all supplied by Dr. V. L. Narayanan, National Cancer Institute (Bethesda, MD). With the exception of CCNU, which was dissolved in absolute ethanol, all drugs were dissolved in culture medium immediately prior to use. Where used, levels of ethanol were less than 1% and non-cytotoxic.

SCE assay. Cells were seeded at 5×10^4/ml in 75 cm^2 flasks and incubated for 24 hr before exposure to various concentrations of drugs for 1 hr. Medium was replaced with fresh medium after centrifugation of cultures for 4 min, 800 rpm at ambient temperature. Cells were then incubated for 48 hr in the presence of 50 μM bromodeoxyuridine (BUdR) (Calbiochem-Behring Corp., La Jolla, CA) in a light-proof box. Cultures were enriched in mitotic cells by incubation for 2 hr with 2×10^{-7} M colchicine (Sigma Chemical Co., St. Louis, MO), washed with PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 12.9 mM Na_2HPO_4, pH 7.4), and resuspended in 10 ml of 0.075 M KCl for 10 min. Cells were fixed three times with 3:1 methanol–acetic acid and dropped onto clean glass microscope slides.

The fluorescent + Giemsa technique of Perry and Wolff [11] was employed for differential staining of sister chromatids, with conditions optimized as suggested by Goto *et al.* [12]. Briefly, preparations were stained in 5 μg/ml aqueous solution of Hoechst 33258 (Sigma) for 15 min, rinsed, then mounted in 0.1 M phosphate buffer, pH 8.0, and the coverslips sealed with rubber cement. Slides were exposed to black light (single black light bulb, GE F8T5 BLB at 6 cm) for 15 min over a 50° water bath, coverslips were removed, and slides were rinsed and stained for 15 min in 2% Giemsa (MCB Manufacturing Chemists, Inc., Gibbstown, NJ) in 0.01 M phosphate buffer, pH 6.8. When dry, the finished preparations were mounted in Permount (Fisher Scientific Co., Fairlawn, NJ), and SCE frequency was determined for at least 500 chromosomes per treatment.

Alkaline elution assay. Cells were seeded at 5×10^4/ml in 75 cm^2 flasks and incubated for 24 hr in the presence of 0.1 μCi/ml [^{3}H]TdR (60 Ci/mmole, ICN Pharmaceuticals Inc., Irvine, CA) and then for a further 24 hr in fresh medium. Cells were suspended at 10^4/ml in 10 ml medium and treated with various concentrations of drug for 1 hr (HN2, PM) or 6 hr (CCNU, CLZ). For repair studies, drug treatment was carried out at intervals, and damage remaining after 1–54 hr was assayed. After incubation with drugs, cells were suspended in 1 ml PBS and exposed to 500 rads from a Caesium source. The procedure for alkaline elution was similar to that described by Kohn *et al.* [13]. Briefly, cells were layered onto 2 μm PVC membrane filters (Millipore Corp., Bedford, MA) and lysed by addition of 5 ml of lysis solution (0.02 M Na_2EDTA, 2% sodium dodecyl sulfate, 0.1 M glycine, pH 10.0), allowed to drip through under gravity; 2 ml of 0.02 M Na_2 EDTA, pH 10.0, was added, containing 0.5 mg/ml Proteinase-K (Sigma) when required, and then DNA was eluted by addition of 20 ml of elution buffer, pH 12.1, pumped at 2 ml/hr, collecting 10×1 hr fractions. Results were calculated as the fraction of ^{3}H-labeled DNA retained on the filter, and cross-link index was calculated as previously described [13], comparing relative retention of treated and control irradiated DNA at Fraction No. 8. Where differences in cross-linking were suspected, regression analysis and a modified *t*-test were applied to establish significance. DNA-protein cross-linking was estimated by subtraction of DNA-interstrand cross-links from total cross-links.

RESULTS

HN2 caused a dose-dependent increase in total cross-linking which was similar in WR and WS (Fig. 1A). DNA-interstrand cross-linking constituted approximately 50% of the total in both cases, the remaining 50% being Proteinase-K-sensitive and hence due to DNA-protein cross-links. At concentrations of PM up to 100 μM, we could detect no cross-linking of either type at 1 or 6 hr, nor could we detect any DNA-interstrand cross-links at 6 hr after treatment with concentrations as high as 1 mM (data not shown). This is in contrast to reports by Erickson *et al.* [14] and Ramonas *et al.* [15] who report significant levels of both DNA-interstrand and DNA-protein cross-links in L1210 cells, reaching a maximum at around 3 and 6 hr respectively. As CENUs take several hours to cause maximal cross-linking [16], we assayed CLZ- and CCNU-induced cross-links after 6 hr. CLZ induced considerably lower levels of DNA-interstrand cross-links than did HN2, again, equivalent in both cell lines (Fig. 1B). We could detect no CCNU-induced DNA-interstrand cross-links even at super-lethal concentrations of drug [5] as high as 1 mM (data not shown). When assayed in the absence of Proteinase-K, there was significantly more cross-linking in WR than in WS after both CLZ ($P < 0.001$) and CCNU ($P < 0.1$), (Fig. 1C and D). In light of the DNA-interstrand cross-linking data (Fig. 1B), this indicates significantly increased CENU-induced DNA-protein cross-links in WR compared to WS.

The ability of cells to repair HN2 and CLZ-induced cross-links was measured over periods of up to 54 hr. After treatment with 1 μM HN2, both cell lines showed a similar rate of removal of HN2-induced DNA-interstrand cross-links, which was almost complete by 24 hr (Fig. 2A). Furthermore, there was no difference in the ability of the two cell lines to remove total cross-links (Fig. 2B), suggesting that DNA-protein cross-links were repaired equally well in WR and WS.

In a similar experiment using 100 μM CLZ, more cross-links were found in WR cells. There was rapid removal of cross-links from 6 to 20 hr in both cell lines (Fig. 2C) after which the lesions were repaired more slowly with complete removal between 48 and 54 hr post-treatment. Considering the difference in initial (6 hr) cross-links, the kinetics of removal of

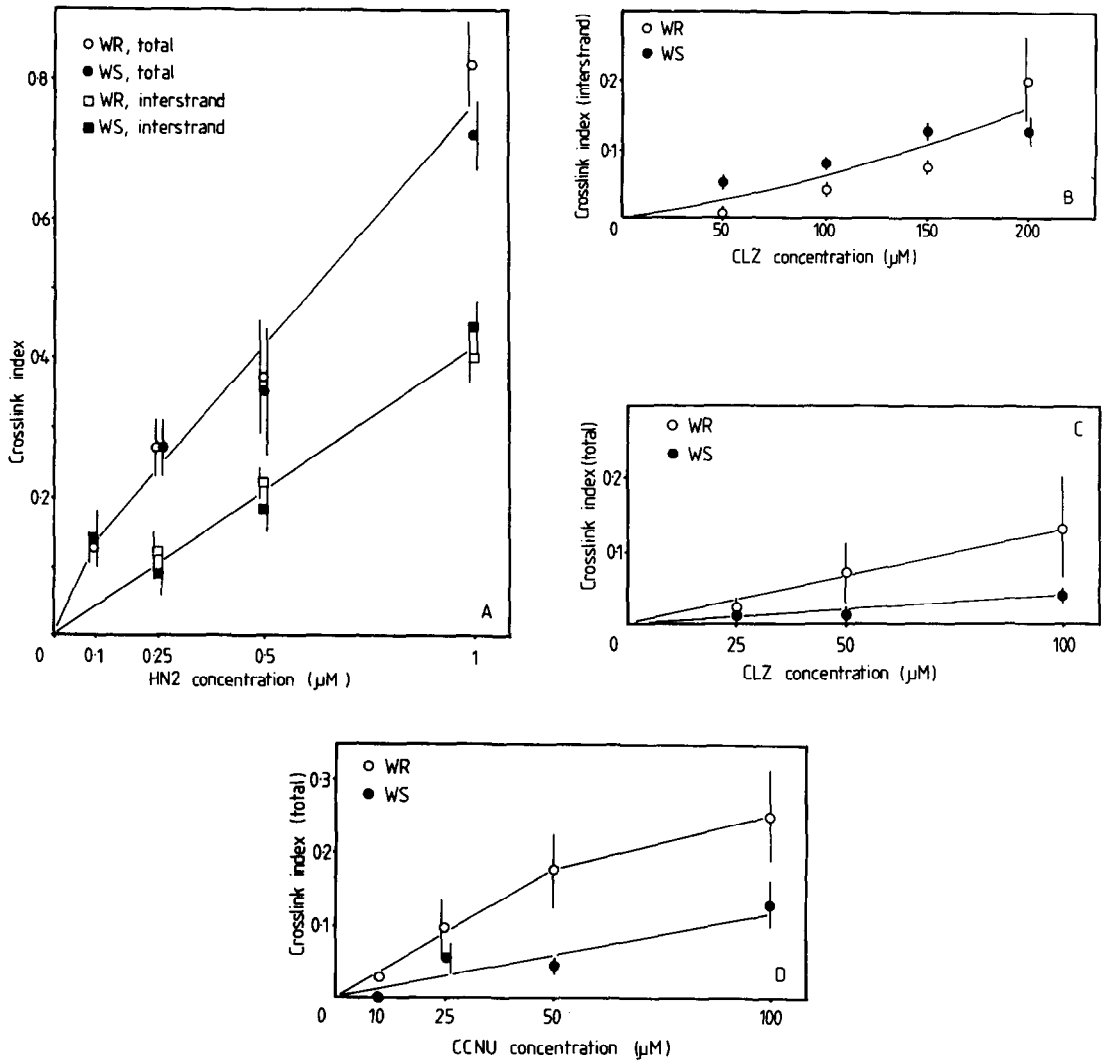

Fig. 1. Cross-linking in WR and WS cells after treatment with various concentrations of HN2 for 1 hr (A), CLZ for 6 hr (B and C) or CCNU for 6 hr (D). Data points were derived from the mean of at least three separate experiments ± S.E. Cross-link indices were calculated as described previously [13].

CLZ-induced cross-links was very similar in the two cell lines.

It was not possible to study DNA-interstrand cross-link repair following CLZ because the concentrations required to cause measurable lesions produced toxicity which was manifested through cytolysis. This was also true for total CCNU-induced cross-links.

SCE analysis was carried out over a range of concentrations for all four drugs. HN2 caused a dose-dependent increase in SCE frequency in both cell lines (Fig. 3A), the magnitude of the effect being greater in WS. At the highest dose tested there were approximately twice as many SCEs in WS as in WR. Qualitatively, this reflects the reduced survival in WS, but HN2 is at least ten times more toxic to WS than to WR [5]. PM caused progressively increased SCE in WS cells at 2.5 and 5.0 μM, but at these drug concentrations there was no measurable increase in SCEs in WR (Fig. 3B).

Neither CLZ nor CCNU caused any increase in SCE frequency in WS cells (Fig. 3, C and D), but both drugs caused significantly increased levels of SCEs in WR cells.

DISCUSSION

In a previous study [5], a comprehensive analysis of the sensitivity of the WR and WS cell lines showed that the WR cells were at least 10- to 15-fold more resistant than the parent cell line to a variety of NMs, yet exhibited an approximately 2- to 4-fold collateral sensitivity to a range of CENUs. It is apparent from the data in Fig. 2 that both cell lines are equally capable of repairing HN2-induced DNA-interstrand and DNA-protein cross-links, as well as CLZ-induced total cross-links. This indicates that a differential in the removal of drug-induced cross-links is not, in itself, an explanation for the resistance of

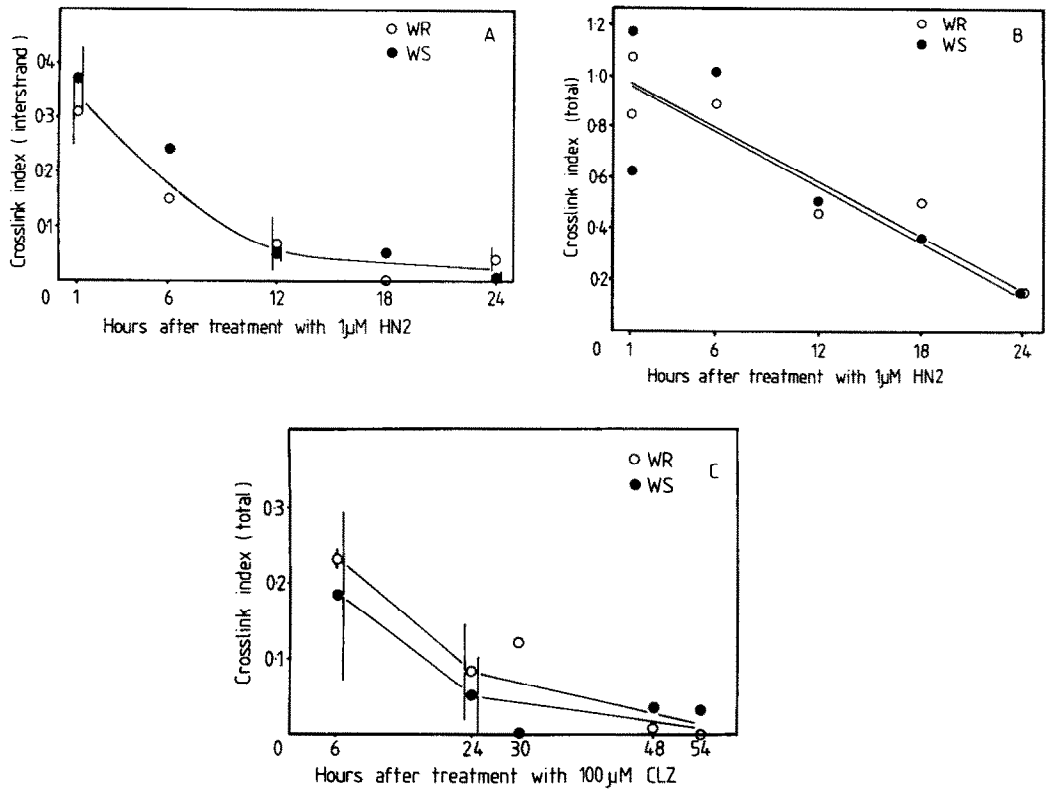

Fig. 2. Removal of cross-links in WR and WS cells after treatment with 1 μM HN2 (A and B) or 100 μM CLZ (C). Data points in (B) were derived from two separate experiments and lines fitted by regression analysis. Points with bars represent mean ± S.E. of three separate experiments.

WR to NMs or the circumvention of this resistance by CENUs.

We have also observed that treatment of cultures with NMs caused increased frequency of SCEs in the WS cells. This is in agreement with several reports relating increased formation of SCEs with drug sensitivity [17–19]. Since there was no difference in mustard-induced DNA-interstrand or DNA-protein cross-link frequency, the lower SCE frequency in WR and the resistance to NMs may correlate with a reduced incidence of monofunctional alkylation products. Reduced alkylation in WR cells may be a reflection of the increase in detoxifying glutathione-*S*-transferases found in this cell line when compared to WS [7]. Furthermore, the absence of detectable cross-links after cytotoxic concentrations of PM may suggest that cross-links are not prerequisites for mustard-induced cell death in these cell lines. This is in contrast to other reports which suggest that PM-induced cross-links can be correlated with cytotoxicity in L1210 cells [14, 15].

Bodell *et al.* [20] reported increased levels of DNA cross-links and SCEs after treatment of rat 9L cells sensitive to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). This established a correlation between induced cross-links, cytotoxicity and SCEs. These findings are consistent with our data for both CLZ and CCNU if we consider DNA-protein cross-linking. However, in the Walker cells, we could detect no DNA-interstrand cross-links after CCNU and no difference in the levels of DNA-interstrand cross-links after CLZ treatment, implying that DNA-interstrand cross-linking of WR or WS by CENUs is, again, less important in cell killing than other drug reactions.

The carbamoylating properties of CENUs [21] and depleted levels of glutathione reductase [6] have already been implicated in overcoming mustard resistance in WR cells. Depletion of glutathione by this mechanism would decrease the ability of the WR cells to detoxify alkylating electrophiles, creating the potential for increased DNA reaction, perhaps of a type favoring subsequent cross-linking to cellular proteins.

Another explanation for increased DNA-protein cross-linking in WR cells after CENUs may lie in the nature of the adducts formed. Several lines of evidence from previous investigations indicate a role for the enzyme guanine-O^6-alkyl transferase (GO6AT) in the removal of chloroethyl monoadducts prior to cross-link formation [4, 22]. Thus, it is possible that WS cells can repair CENU-induced monoalkylation damage, whereas WR cells have a depleted capacity for this function. Although the lack of differential DNA-interstrand cross-linking contradicts this idea, the enzyme may play a role in the prevention of CENU-induced DNA-protein cross-links in these cells, a lesion which does show

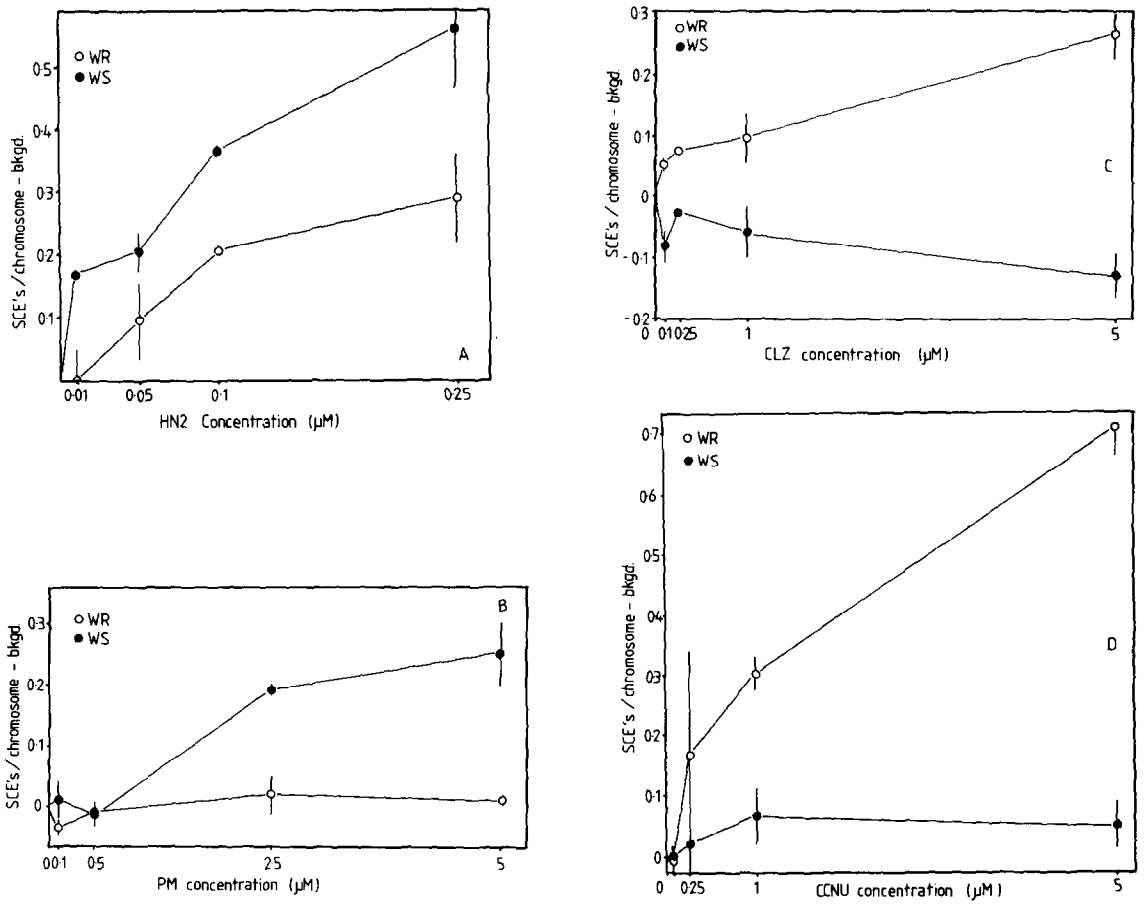

Fig. 3. Increase in SCE frequency (over that induced by 50 μM BUdR alone) in WR and WS cells after treatment for 1 hr with HN2 (A), PM (B), CLZ (C) or CCNU (D). Each point represents the mean ± S.D. of up to five separate counts totalling at least 500 chromosomes in each. Background levels of SCE following BUdR treatment were: WS, 0.26 ± 0.1 SCE/chromosome, and WR, 0.12 ± 0.06 SCE/chromosome.

correlation with both cytotoxicity and SCE-induction. It is also interesting to note that HN2-induced damage has been shown not to be susceptible to this enzyme [23, 24], and we observed similar, high levels of HN2-induced cross-links in both the WS and WR.

The results of the study presented indicate that a complex series of correlations exist for cytotoxicity, DNA interaction and SCE in the Walker cell lines following exposure to NM or CENU. The following conclusions about these cells may be drawn from our observations: (i) There is no difference in the ability of WR and WS cells to remove HN2 or CLZ-induced cross-links. (ii) Equal amounts of DNA-interstrand cross-links after HN2 or CLZ and no detectable DNA-interstrand cross-links after CCNU or PM suggest that DNA-interstrand cross-linking is not the crucial factor in the determination of the differential cytotoxicity in these cells. (iii) With respect to drug concentration and cytotoxicity, CENUs cause fewer cross-links than HN2, possibly due to the function of GO6AT, the levels of which may be reduced in WR cells. (This would be consistent with the previous report that selection of the WR population resulted in the reduced activity of another enzyme, glutathione reductase [6].) (iv) NM-induced adducts that are responsible for causing DNA-interstrand or DNA-protein cross-links do not correlate directly with those causing SCEs and cell death. (v) CENU-induced adducts that are responsible for cell killing, SCEs and DNA-protein cross-links do not correlate directly with those causing DNA-interstrand cross-links.

The apparent lack of a simple correlation between cytotoxicity, crosslinking and SCE induction in the Walker cells is distinct from other data for other cell lines [14, 15, 20]. This may reflect differences in the selection and maintenance of the WR cells or may be the consequence of degrees of population heterogeneity, although previous karyotypic characterization suggested that WR cells had a high degree of homogeneity with respect to certain chromosome markers [9]. These observations in no way question the validity of previous studies, but merely extend the concept that the acquisition of drug resistance to nitrogen mustards need not be the result of a single genotypic and/or phenotypic alteration.

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