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Selection of Nitrogen Mustard Resistance in a Rat Tumor Cell Line Results in Loss of Guanine-O^6-Alkyl Transferase Activity

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SUMMARY

Cell killing, DNA-interstrand crosslinks, and DNA-protein crosslinks were assayed in nitrogen mustard-resistant Walker 256 carcinoma (WR) cells and the parent cell line (WS) after treatment with 5-[3-(2-chloroethyl)-1-triazenyl]imidazo-4-carboxamide (MCTIC). The WR cells, which also express collateral sensitivity to chloroethylnitrosoureas, were approximately twice as sensitive to the cytotoxic effects of MCTIC as were WS cells. Following treatment with 100 μM MCTIC, there was a rapid accumulation of both DNA-interstrand and DNA-protein crosslinks in the WR cell line, which reached a maximum at 6 and 12 hr, respectively. There was considerably less crosslinking in the WS cells and both cell lines were proficient in repairing most of the crosslinks by 24 hr. Measurement of guanine-O^6-alkyl transferase activity showed the enzyme to be present in WS but not in WR cells. These data indicate that the collateral sensitivity of nitrogen mustard-resistant WR cells to chloroethylating drugs is in part due to the loss of guanine-O^6-alkyl transferase activity which is present in the parent line.

A serious clinical problem encountered in the chemotherapy of human tumors is the development of drug resistance within the malignant cell population. This is particularly important when resistance is not confined to the original drug used. This problem can be conveniently studied using tumor models in cell culture. Patterns of cross-resistance (1–3) or its absence (1, 4–6) have been described although very rarely have the precise mechanisms been elucidated. A valuable tumor model is represented by two Walker 256 mammary carcinoma cell lines expressing resistance (WR) and sensitivity (WS) to NMs (7). NM resistance has been partially attributed to increased levels of glutathione-*S*-transferase (8) and DNA crosslinking studies suggest that DNA is not the only target which determines cytotoxicity (9). The WR cell line shows collateral sensitivity to several CENUs, and this has been attributed to the cells having reduced levels of glutathione reductase (10), an enzyme important in maintaining the cellular thiol:disulfide balance, which is susceptible to inactivation through carbamoylation by CENUs (11). However, CENUs are no more toxic to WR than *N,N'*-bis(*trans*-4-hydroxy-cyclohexyl)-*N'*-nitrosourea, a nitrosourea with carbamoylating but not alkylating activity (10), inferring that, whereas the alkylating properties of NMs are toxic to WS, those of CENUs are less so.

We now report the cytotoxic and DNA-damaging effects of MCTIC on both WR and WS cells. MCTIC was selected as it closely mimics the DNA-damaging effects of CENUs (12, 13) yet does not carbamoylate cellular proteins (14). MCTIC thus allows us to study the importance of alkylation to the phenomenon of collateral sensitivity without any interference by carbamoylation. The results indicate that WR cells are collaterally sensitive to MCTIC and that these cells, in acquiring resistance to NMs, have lost GO6AT activity.

Materials and Methods

Cell Cultures. Derivation of Walker 256 rat mammary carcinoma cells resistant (WR) from the parent, sensitive (WS) line has been previously described (7). Both lines were maintained as static suspension cultures in Dulbecco's minimal essential medium containing 4.5 g/liter of glucose (Biotech Research Laboratories, Rockville, MD), 4 mM L-glutamine, 50 units/ml of penicillin, 50 μg/ml of streptomycin (MA Bioproducts, Walkersville, MD) supplemented with 10% fetal calf serum (KC Biological, Kansas City, KS) under 5% CO_2. Resistance of WR is maintained by biannual exposure to 20 μg/ml of chlorambucil, followed by serial passage in the usual way. HT-29 human colon carcinoma cells were maintained as described elsewhere (15).

Drug Treatment. MCTIC was a gift from Professor M. F. G. Stevens, Aston University, England. MCTIC was dissolved in dimethyl

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ABBREVIATIONS: NM, nitrogen mustard; CENU, chloroethylnitrosourea; EDTA, ethylenediaminetetraacetic acid; GO6AT, guanine-O^6-alkyl transferase; MCTIC, 5-[3-(2-chloroethyl)-1-triazenyl]imidazo-4-carboxamide MNU, 1-methyl-1-nitrosourea.

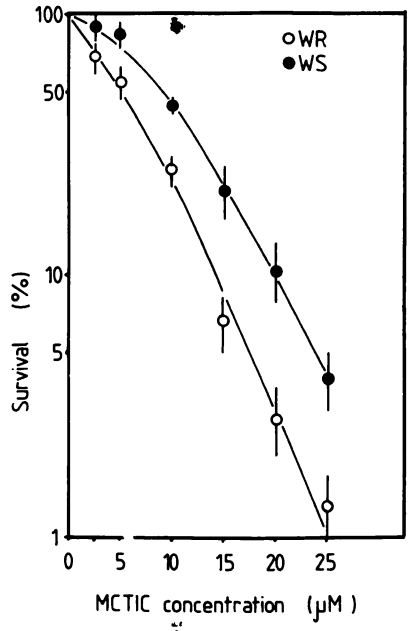

Fig. 1. Effect of MCTIC on survival of WR (○) and WS (●). Points represent mean and standard deviation of triplicate experiments each involving quadruplicate sets of plates. Cells were treated for 2 hr in suspension in culture medium (Dulbecco's minimal essential medium, 4.5 g/liter of glucose, 4 mM L-glutamine, 10% fetal calf serum antibiotics) and plated in 0.3% noble agar as previously described (7). Colonies containing 50 or more cells were counted after incubation at 37°, 5% CO_2 for 7 days.

sulfoxide immediately before treatment of cells. Concentration of solvent in control or treated cultures was always less than 2%.

Alkaline elution assay. The procedure for alkaline elution was similar to that of Kohn *et al.* (16) and is described in detail elsewhere (9). For analyses of interstrand cross-links, cells were lysed on 0.8-μm-pore size polycarbonate (Nucleopore) filters with 2% sodium dodecyl sulfate, 0.025 M EDTA, pH 10.0, and proteinase K (0.5 mg/ml) and were eluted at 2 ml/hr with tetrapropylammonium hydroxide/EDTA (pH 12.1) containing 0.1% sodium dodecyl sulfate. For assay of interstrand crosslinks, the cells cooled in ice were irradiated with 300 R ^{137}Cs γ-rays. Internal standards were [^{3}H]thymidine-labeled L1210 cells irradiated with 300 R in the cold. Interstrand crosslink index is defined as

$$\left(\frac{1 - R_0}{1 - R_1}\right)^{1/4} - 1$$

where R_0 and R_1 are relative retention of [^{14}C]DNA at 25% retention of [^{3}H]DNA; corrections for strand breaks were applied.

For assay of total DNA crosslinks, cells were irradiated with 300 R ^{137}Cs γ-rays in the cold. Cells were lysed on 2 μM pore size polyvinylchloride filters with 2% sodium dodecyl sulfate, 0.025 M EDTA, pH 10.0. The detergent was then washed away with 5 ml of 0.02 M disodium EDTA, pH 10.0. Elution was with tetrapropylammonium hydroxide/EDTA, pH 12.1. Internal standards were [^{3}H]thymidine-labeled L1210 cells irradiated with 300 R.

The effects of MCTIC on glutathione reductase were assayed using methodology described elsewhere (10) and based on the method of Mize and Langdau (17).

Assay of GO6AT activity. Approximately 5 × 10^8 cells were harvested and prepared for analysis, as described by Karran *et al.* (18). Fifty-μl aliquots of cell extracts were incubated with [^{3}H]MNU-alkylated DNA prepared as described (18) in 100 μl of buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol, for 1 hr at 37°. Reaction was stopped by boiling with sodium dodecyl sulfate and the resultant mixture was electrophoresed on a 15% acrylamide gel according to the method of Bonner *et al.* (19). The resultant gel was dried and autoradiographed for 4 weeks at −70°. This procedure was sensitive enough to detect the presence of GO6AT.

Results

Survival of WR and WS cells after treatment with various concentrations of MCTIC is shown in Fig. 1. The WS cells (ID_{50} = 8.5 μM) are approximately 1.7 times more resistant than the WR cells (ID_{50} = 5 μM). This compares favorably with a figure of approximately 1.2-fold reported by Horgan and Tisdale (14) using the parent compound mitozolomide.

The kinetics of formation and loss of MCTIC-induced crosslinks are shown in Fig. 2. The induction of DNA-interstrand crosslinks in WS peaked at 12 hr but in WR reached a maximum at 6 hr, with more than 3 times the number of crosslinks seen in WS (Fig. 2A). In both cell lines, total crosslinking also reached a maximum at 12 hr, at which time there were about

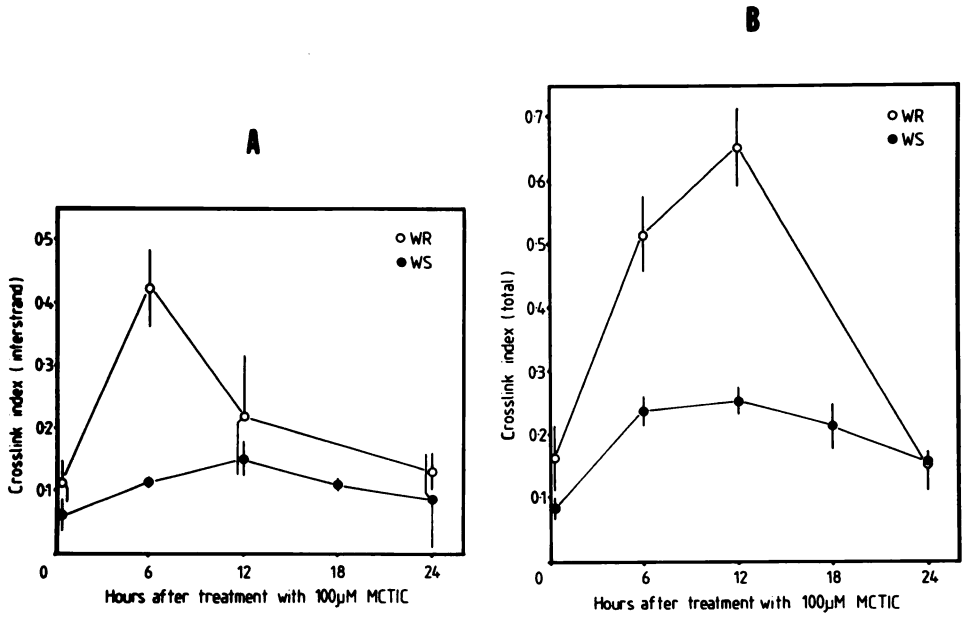

Fig. 2. DNA interstrand (A) and total (B) crosslinking in WR (○) and WS (●) after 2 hr treatment with 100 μM MCTIC followed by incubation in drug-free media for the times indicated. Each point represents the mean and standard error of three separate measurements. The procedure for alkaline elution was adapted from that of Kohn *et al.* (16) and is similar to that previously used by us (9).

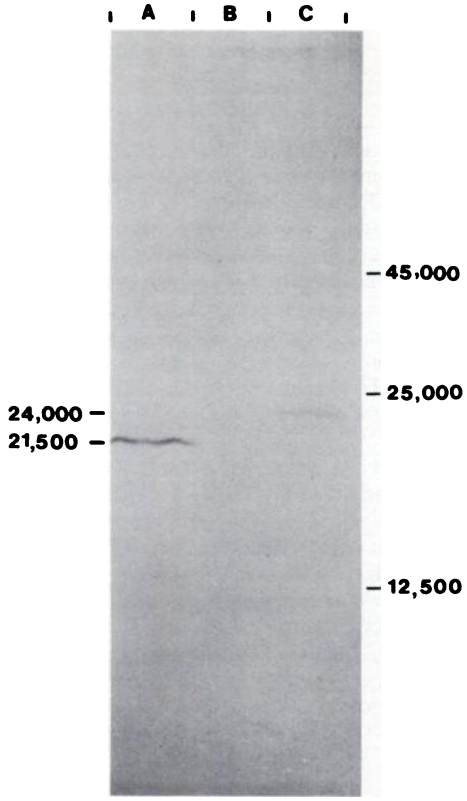

Fig. 3. Gel autoradiograph showing the ^{3}H-methylated GO6AT derived from HT-29 human colon carcinoma cells (*lane* A), WR cells (*lane* B), and WS cells (*lane* C). Numbers to the *right* of the gel indicate the positions and molecular weight of the standards used, those to the *left* indicate the molecular weight of the observed radioactive protein band. Approximately 5×10^8 cells were harvested and prepared for analysis as described by Karran *et al.* (18). Fifty-μl aliquots of cell extracts were incubated with [^{3}H]MNU-alkylated DNA, prepared according to the method of Day *et al.* (20), in 100 μl of buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol, for 1 hr at 37°. Reaction was stopped by boiling with sodium docecyl sulfate and the resultant mixture was electrophoresed on a 15% acrylamide gel according to the method of Bonner *et al.* (19). The resultant gel was dried and autoradiographed for 4 weeks at −70°.

2.5 times more total crosslinks in WR than in WS (Fig. 2B). Despite more rapid formation of both types of crosslinks in WR cells, both cell lines were capable of repair and had achieved similar reduced levels by 24 hr.

The removal of an alkyl group from the O^6 position of guanine in calf thymus DNA previously alkylated with [*methyl*-^{3}H] MNU can be measured as follows. The DNA substrate is incubated with cell extracts of each cell line and then gel electrophoresis is performed according to the procedure of Bonner *et al.* (19). The presence of a radioactive protein band in the gel autoradiograph is indicative of the presence of GO6AT activity. WR cells were found to have no GO6AT activity (Fig. 3, lane B), whereas WS cells had GO6AT activity (Fig. 3, Lane C). These results are consistent with the fact that WR cells are Mer$^-$ and WS cells are Mer$^+$. Fig. 3, lane A, shows the GO6AT activity present in a cell line, previously shown to be Mer$^+$ (20), the HT-29 human colon carcinoma. The molecular weight of HT-29 GO6AT was found to be 21,500 and is in close agreement with previous reports of 21,000–22,000 for human lymphoid cells (21) and 23,000 or 24,000 for human liver or HeLa cells, respectively (22). The 24-kDa molecular weight band present in lane C (WS) but not lane B (WR) of Fig. 3 represents the GO6AT from the rat mammary carcinoma and is somewhat larger than a previous estimate for rat liver GO6AT (23). This confirms the suspicion that WR cells lacked GO6AT activity which was present in the parent cell line.

Discussion

Cellular resistance to NMs in WR cells has been acquired at the expense of increased sensitivity to classes of drugs which possess carbamoylating and/or O^6-guanine-alkylating properties. We have now identified several mechanisms contributing to those features of drug resistance and collateral sensitivity. The development of NM resistance can be related to increased levels of glutathione-*S*-transferases which, in still more resistant sublines, is further increased (8). Since we cannot readily identify any differential DNA reaction of NMs in these two cell lines (9), then a non-DNA target for cytotoxicity seems plausible. Since differences in both cyclic nucleotide phosphodiesterases and phosphorylation of nuclear matrix proteins and associated enzymes have been found (24–27), then potential targets for NM-induced toxicity may emerge from these groups of proteins.

Previous observations have suggested that a reduced level of glutathione reductase in the resistant cells leaves them more susceptible to the lethal effects of thiol:disulfide imbalances brought about by carbamoylation of the enzyme (10). However, the present study shows that MCTIC, which did not inhibit glutathione reductase (data not shown), yet was capable of alkylating at the O^6 position of guanine (12–14), was also more toxic to the WR cells (Fig. 1). MCTIC is a bifunctional alkylating agent which has previously been shown to crosslink DNA (12, 13). The increase in DNA interstrand crosslinking in WR compared to WS after MCTIC (Fig. 2A) is characteristic of the response of Mer$^-$ compared with Mer$^+$ cells described previously (12, 13). Normal human cells, as well as the majority of human tumor cell strains, possess a GO6AT activity which removes methyl, ethyl, and perhaps chloroethyl adducts from guanine O^6 positions (20). Such cells are described as possessing the Mer$^+$ phenotype (20). In this study we have shown that WR cells lack GO6AT activity and thus possess the Mer$^-$ phenotype, whereas WS cells are of the Mer$^+$ phenotype. The rapid formation of DNA-interstrand crosslinks in WR cells reflects the inability to remove the monoadduct prior to crosslink formation (28, 29). They appear, however, to be able to repair DNA-interstrand crosslinks once formed as evidenced by crosslink removal 12–18 hr after drug treatment. This type of repair activity is probably unrelated to GO6AT activity and may be due to excision repair.

The correlation between increased GO6AT activity and reduced DNA-interstrand crosslinks indicates that GO6AT plays a role in the prevention of DNA-interstrand crosslinks as previously suggested (28, 29). However, it is still not clear precisely which is the cytotoxic lesion, since recent observations suggest that merely the persistence of monofunctionally chloroethylated guanines is sufficient to cause cellular toxicity and that DNA-interstrand crosslinks may be less important (15). It is also possible that the DNA-protein crosslinks were important lesions since they were more persistent in WR than the DNA-interstrand crosslinks (Fig. 2).

In acquiring resistance to NMs, the WR cells experienced a reduction in chromosome number (7) and it may well be that the gene(s) coding for GO6AT was (were) lost. The reduction

in glutathione reductase activity may be explained if a proportion of the relevant genetic material was lost. At the chromosomal level it is interesting to note that there is a reduction in the aneuploid number from 62 in WS to 54 in WR (7, 27). In addition, there are a number of C-banding differences between the cell lines.[3] The absence of GO6AT and the depletion of glutathione reductase (10) in WR cells could presumably be a function of this reduction in genetic information and may provide information about the chromosomal locations of the genes coding for these enzymes.

In conclusion, we have defined a mechanism by which a cell line expressing resistance to one class of agents exhibits collateral sensitivity to another related group. More specifically, the acquisition of resistance to NMs occurred at the expense of increased sensitivity to CENUs, due both to a reduction in glutathione reductase (10) and a loss of GO6AT activity.

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[3] B. Asp, A. Billstrom, and K. D. Tew, unpublished data.

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