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STUDIES ON THE MECHANISM OF RESISTANCE TO MITOMYCIN C AND PORFIROMYCIN IN A HUMAN CELL STRAIN DERIVED FROM A CANCER-PRONE INDIVIDUAL

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Abstract—The mechanism of aerobic resistance to the quinone-containing anti-tumour agents mitomycin C (MMC) and porfiromycin (PM) has been investigated using non-transformed human cells. One of the cell strains used (3437T) was derived from an afflicted member of a cancer-prone family. This cell strain had been shown previously to be six times more resistant to the cytotoxic effects of these agents under aerobic but not hypoxic conditions when compared to a cell strain derived from an unrelated, normal donor (GM38). Differences could not be detected in the ability of cell sonicates prepared from either cell strain to produce alkylating species under aerobic conditions using a 4-(*p*-nitrobenzyl)pyridine assay. However, using ^{3}H-labelled PM to monitor rapid drug uptake and subsequent accumulation due to drug metabolism, results were obtained indicating that the resistant cell strain (3437T) was deficient in an enzymatic pathway capable of metabolizing these compounds under aerobic but not hypoxic conditions. Dicumarol, an inhibitor of the quinone reductase DT-diaphorase (EC 1.6.99.2), decreased aerobic drug accumulation and cytotoxicity in the control cell strain, but did not alter the lack of accumulation noted in the resistant cell strain. Under hypoxic conditions, dicumarol increased cytotoxicity and drug accumulation in both cell strains. The mechanism of this enhanced cytotoxicity remains unclear. These results suggested that the resistant cells were deficient in the enzyme DT-diaphorase, a potential activator of PM. Enzymatic assays confirmed this and revealed no alterations in cytochrome P450 reductase (EC 1.6.2.4) activity or glutathione content. No protein characteristic of DT-diaphorase was detected in the resistant cell strain using a polyclonal rabbit-anti-rat antibody raised against this enzyme. Southern blot analysis using a rat DT-diaphorase cDNA probe demonstrated differences between the normal and resistant cell strains in the restriction fragment patterns. The present results are consistent with the hypothesis that decreased DT-diaphorase levels are causally associated with PM and MMC resistance in these cells under aerobic exposure conditions.

Bioreductive alkylating agents, such as the quinone-containing compounds mitomycin C (MMC§) and porfiromycin (PM), have been investigated for their potential as adjuncts to radiation therapy in the treatment of solid tumours [1, 2]. This interest arises from their preferential toxicity towards radioresistant hypoxic populations of cells which may survive radiation treatment and lead to local recurrence of the tumour [3–5]. The basis of this preferential toxicity is as follows. Both one- and two-electron reduction pathways have been proposed to generate reactive intermediates of MMC, the semiquinone and hydroquinone, which, after subsequent rearrangements, can alkylate DNA. The one-electron reduction pathway is particularly sensitive to oxygen due to the sensitivity of the semiquinone to back-oxidation. While alkylation is believed to be most important for cytotoxic activity, generation of reactive oxygen species from oxidation of reduced intermediates may also contribute to the toxicity of this class of compounds under aerobic exposure conditions [6, 7].

By altering specific aspects of this metabolic activation scheme, other factors such as ascorbic acid [8], caffeine [9], acidic pH [10] and dicumarol [11] may also alter the toxicity of these compounds. In particular, dicumarol (DIC), an inhibitor of the quinone reductase NAD(P)H: (quinone-acceptor) oxidoreductase (EC 1.6.99.2) (also known as DT-diaphorase), has been shown to decrease aerobic cytotoxicity in rodent cell lines [12, 13] and a human cell strain [14]. This is consistent with an important role for DT-diaphorase in drug activation under aerobic conditions. Dicumarol has also been shown to increase the toxicity of these drugs under hypoxic conditions. This may be due to inhibition of an enzyme other than DT-diaphorase [13, 15] and/or alteration of cellular redox state.

Previously, we have studied the sensitivity of two human skin fibroblast strains to MMC and PM [14]. One strain was derived from a patient who had

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§Abbreviations: MMC, mitomycin C; PM, porfiromycin; DIC, dicumarol; α-MEM, minimum essential medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; PNBP, 4-(*p*-nitrobenzyl)pyridine; TCA, trichloroacetic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; AZQ, diaziquone; and GSH, glutathione.

developed multiple cancers and belonged to a family with an increased incidence of cancer. This cell strain was found to be resistant to both agents under aerobic conditions. Decreased cross-linking of DNA was also observed in the resistant cell strain under aerobic conditions using the alkaline elution technique [16]. However, the sensitivity of the resistant cell strain was equivalent to that observed in a normal control cell strain if exposures were conducted under hypoxic conditions. DIC did not alter the aerobic toxicity in the resistant cells but did protect the control cells from MMC and PM toxicity. Preliminary data demonstrated decreased levels of DT-diaphorase in this cell strain, suggesting that the aerobic resistance could be related to alterations in an enzyme(s) involved in the activation of these agents under aerobic conditions. The resistant cells did not demonstrate increased P-glycoprotein expression [14].

In the present work, the normal and MMC-resistant cell strains have been examined further in an attempt to determine the molecular basis for resistance to MMC and PM under aerobic conditions. Some possible explanations include the following: (1) alteration in the metabolic activation of these bioreductive alkylating agents [8, 17, 18]; (2) decreased cellular accumulation and/or increased efflux of drug [19]; and (3) increased levels of intracellular protective agents such as glutathione [20, 21]. These possible mechanisms of resistance have been investigated in the present work, with particular emphasis on the role that DT-diaphorase may play in determining cellular response.

MATERIALS AND METHODS

Cells. Previous publications have described the cell strains used in these experiments and their maintenance [14, 16]. The GM38 cells were derived from skin explants obtained from a healthy 9-year-old female and the 3437T cells from a female who had developed two malignancies and belonged to a family prone to multiple polyposis and sarcomas. Monolayers of cells were grown in 175 cm^2 polystyrene tissue culture flasks (Nunclon, Denmark) in growth medium consisting of α-minimum essential medium (α-MEM) and 10% fetal bovine serum (Bocknek Laboratories, Canada). Cells were sub-cultured in 1:4 dilutions once confluent, and cells between passage numbers 20 to 28 were used for all experiments. Cell number and volume were determined with a cell volume analyzer attached to an electronic particle counter (constructed at the Ontario Cancer Institute, Toronto, Canada).

Chemicals and reagents. PM (a gift from the Upjohn Pharmaceutical Co., Kalamazoo, MI, U.S.A.) and MMC (Boehringer Mannheim, West Germany) were reconstituted with sterile, deionized, distilled water and checked spectrophotometrically to determine concentrations. Dicumarol (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved with equimolar amounts of NaOH in sterile, deionized, distilled water. [^{3}H]PM (sp. act. 36 mCi/mmol) was a gift from Dr. A. Sartorelli (Yale University, New Haven, CT, U.S.A.). The radiolabel was incorporated in the methyl group on the aziridine ring. The polyclonal rabbit-anti-rat DT-diaphorase IgG and the pDTD55 plasmid containing the rat quinone-reductase clone were provided by Dr. C. B. Pickett, Merck-Frost Center for Therapeutic Research, Kirkland, Quebec, Canada. All other chemicals and reagents were obtained from standard sources.

Alkylation assay. An alkylation assay using 4-(*p*-nitrobenzyl)pyridine (PNBP) to detect reactive alkylating species in cell sonicates was performed as described previously by Kennedy *et al.* [17]. The following modifications were made to the published procedure. The reaction mixture contained 2.5 mg protein. Prior to the addition of drug, the reaction mixture was held on ice and exposed to flowing gas containing either 5% CO_2:95% air or 5% CO_2:balance N_2:<10 ppm O_2 for 55 min with occasional shaking. After the addition of drug, the reaction was allowed to proceed for 1 hr at 37°.

Accumulation and efflux of [^{3}H]*PM.* The method of assessing drug accumulation and efflux has been described previously by Keyes *et al.* [22]. Briefly, cells were exposed to radiolabelled drug for various periods of time and sedimented through oil; then cell-associated radioactivity was determined. The only differences from the published technique were that samples of cell suspension were sedimented through 1 mL of an oil–phthalate preparation (dibutylphthalate:vegetable oil, 80:20), and liquid scintillation counting was done in 10-mL volumes of Aquasol.

Cell survival assays. Culture flasks containing approximately 5×10^6 exponentially growing cells were harvested with a 0.25% trypsin solution. Cells were pelleted by centrifugation at 240 *g* for 5 min and resuspended in growth medium to a final cell density of 1×10^6/mL. Exposures of cell suspensions were conducted as described previously [8].

Glutathione assay. Glutathione (GSH) levels were assayed by the method of Teitze [23] as modified by Bump *et al.* [24]. No alterations were made in the published procedure.

DT-diaphorase assay. DT-diaphorase assays were performed by the procedure of Ernster [25] as modified by Benson *et al.* [26]. No modifications were made to this procedure.

NADPH–cytochrome P450 reductase assay. Levels of cytochrome P450 reductase (EC 1.6.2.4) were determined using the procedure of Strobel and Dignam [27]. No modifications were made to this procedure.

Protein immunoprecipitation. Subconfluent, exponentially growing cells in monolayer cultures were washed twice with α-MEM minus methionine and incubated for 1 hr at 37° in similar medium. The medium was removed and replaced with 1 mL methionine-free α-MEM containing 0.2 mCi (>800 Ci/mmol) [^{35}S]methionine (Amersham International, Oakville, Canada), and the cells were incubated for 2 hr. This medium was aspirated, the monolayers were washed with 1.0 mL of cold phosphate-buffered saline (PBS), and 1.0 mL of cold lysis buffer [1% Nonidet P-40, 150 mM NaCl, 20 mM Tris, 0.5 mM phenylmethylsulfonyl fluoride (pH 8.0)] was added, after which the monolayers were left on ice for 30 min. The lysate and cell debris

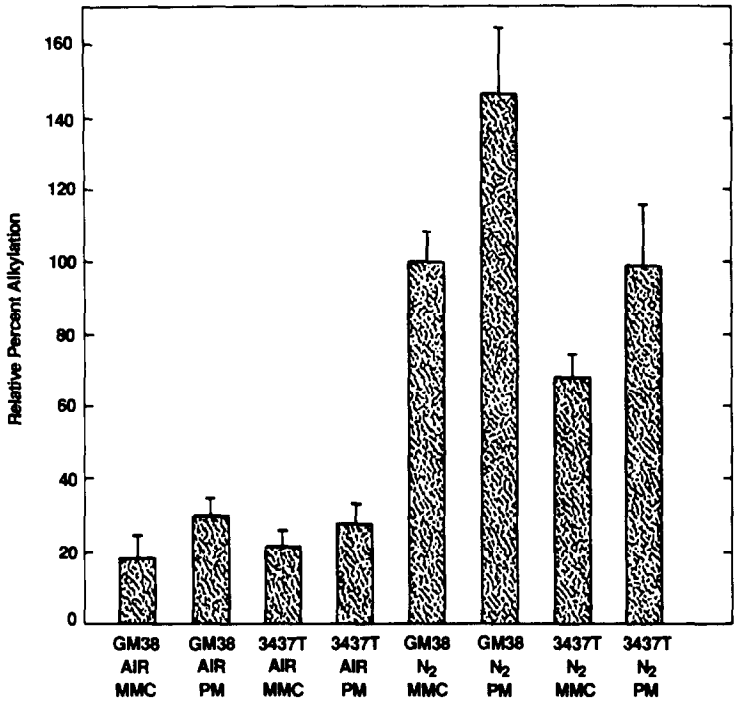

Fig. 1. Comparative measurements of the formation of alkylating species by GM38 and 3437T cell sonicates with either MMC or PM (0.3 mM) under aerobic and hypoxic conditions for 1 hr. Results are expressed relative to the formation of alkylating species observed after a 1-hr exposure of GM38 to MMC under hypoxic conditions (100%, O.D. = 0.070). All results have been corrected for negligible background alkylation which occurs in the absence of drug. Values are means ± SD, N = 5.

were transferred to Eppendorf tubes and microfuged for 4 min; then the supernatant was collected. Twenty microliters (1 μg) of non-specific IgG2a was added and the mixture incubated for 12 hr at 4°. To this precleared preparation, 500 μL of a 10% suspension of washed *Staphylococcus aureus* protein A (SAC, "Pansorbin", Calbiochem, Toronto, Canada) was added, the mixture was incubated for 15 min on ice and then microfuged for 2 min, and the supernatant was recovered. Samples (10 μL) of each reaction were precipitated with TCA, and the amount of radioactivity was determined by scintillation counting as described previously. Volumes of supernatant equivalent to 10^7 acid-precipitated cpm were added to 400 μL of NET-GEL buffer [150 mM NaCl, 5 mM EDTA (pH 8.0), 50 mM Tris (pH 7.4), 0.25% gelatin, 0.02% NaN_3, 0.05% Nonidet P-40]. Either 50 μL of rabbit-anti-rat DT-diaphorase IgG [28] or 40 μL of non-specific human IgG was added to each sample and incubated 1 hr at 4°. Following this, 50 μL of prewashed SAC was added and the mixture was incubated for 15 min on ice. The immune complex of SAC–antibody–protein was pelleted by centrifugation and washed twice with 1 mL of NET-GEL buffer. The protein was eluted from this complex by resuspending the pellet in 30 μL of a protein sample buffer [100 mM dithiothreitol, 2.0% sodium dodecyl sulfate, 10% glycerol, 25 mM Tris (pH 6.8), 0.1% bromphenol blue] and heating at 70° for 10 min. After centrifuging, the supernatant was recovered and the immunoprecipitated polypeptides were subjected to electrophoresis on a 10% SDS–polyacrylamide gel. Gels were visualized by autoradiography on Kodak XAR-5 film.

Southern analysis. Ten micrograms of DNA isolated from cell strains 3437T and GM38 were digested to completion with restriction enzymes and subjected to electrophoresis on a 0.6% agarose gel. This DNA was transferred to nitrocellulose filters overnight using the method of Southern [29] as described by Dulhanty *et al.* [30]. Radioactive "probe" DNA was generated using a plasmid containing the rat quinone-reductase clone pDTD55 [31].

RESULTS

Possible differences in the abilities of cell strains 3437T and GM38 to generate reactive alkylating species were investigated using the PNBP chemical assay. Formation of alkylating species was linear over the period studied for the conditions of the present experiments, and all results were corrected for background alkylation which occurred in the absence of either drug or cell sonicate [8]. Sonicated preparations of either cell strain GM38 (from normal individual) or 3437T (from cancer-prone individual) activated MMC and PM and produced alkylating species under both hypoxic and aerobic conditions (Fig. 1) with the generation of reactive species being considerably greater under hypoxic compared to

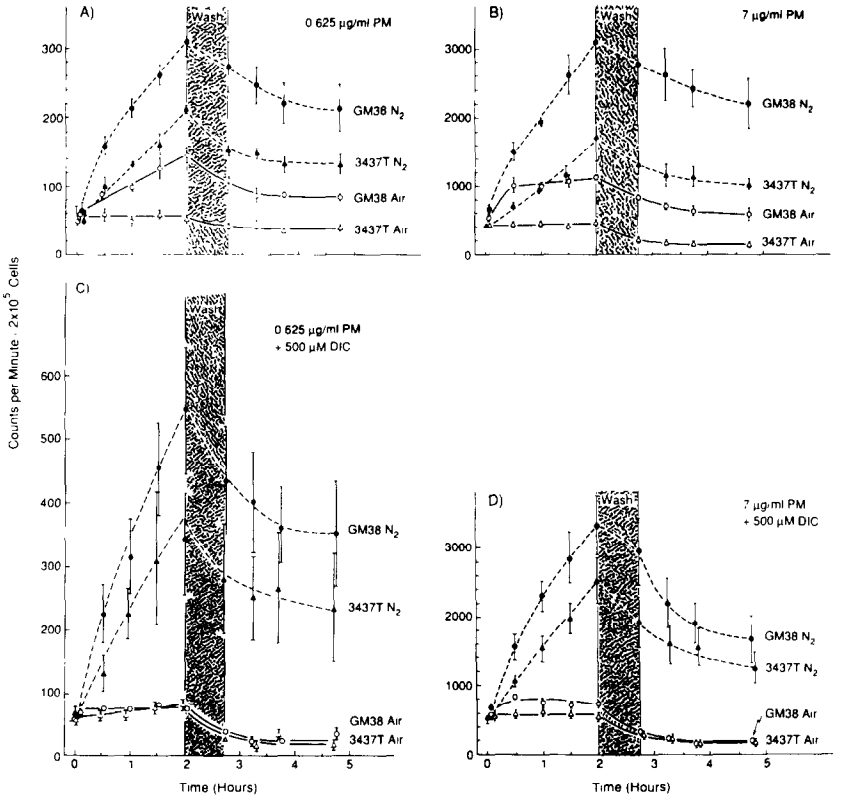

Fig. 2. Uptake and efflux of [^{3}H]PM in suspension cultures of GM38 and 3437T cells under aerobic and hypoxic conditions at low (0.625 μg/mL) (A) and high (7.0 μg/mL) (B) concentrations of PM. The effect of DIC (500 μM) on uptake and efflux of [^{3}H]PM at low (C) and high (D) drug concentrations is also shown. The shaded area corresponds to the period during which cells were washed and resuspended in drug-free medium. Each point is the mean of three experiments ± SEM. All results have been corrected for negligible background.

aerobic conditions. There was no difference between generation of alkylating species by MMC and PM under aerobic conditions. However, equimolar concentrations of PM resulted in greater amounts of alkylation than MMC under hypoxic conditions. In this assay no difference could be detected between the abilities of GM38 and 3437T sonicates to generate reactive alkylating species under aerobic conditions. Under hypoxic conditions 3437T sonicates produced less alkylation than GM38 sonicates.

Studies with tritiated PM demonstrated that the sensitive GM38 cells accumulated drug more rapidly than resistant 3437T cells under all conditions (Fig. 2). As noted previously by Keyes *et al.* [22] using EMT6 rodent cells and Pan *et al.* [32] using HCT 116 human colon carcinoma cells, very rapid uptake of drug occurred almost immediately after drug addition. The amount of this rapid uptake was dependent on the concentration of drug but was not influenced by the presence or absence of oxygen. At low (0.625 μg/mL) and high (7.0 μg/mL) drug concentrations under aerobic conditions, cell-associated radioactivity in the resistant strain 3437T did not increase during the 2-hr exposure period beyond that obtained immediately after drug addition. Levels were considerably higher during the exposure period under aerobic conditions in the normal strain GM38. At lower drug concentrations (0.625 μg/mL) drug continued to accumulate linearly with time over 2 hr. At high drug concentrations (7.0 μg/mL) (Fig. 2B) in air a plateau in accumulation was observed in GM38 cells within 0.5 hr of drug addition. At both low and high concentrations of PM under hypoxic conditions (Fig. 2, dashed lines) cell-associated radioactivity was greater with the normal strain (GM38) than the resistant strain (3437T). Under these conditions both strains accumulated drug linearly after the initial 30 min and both accumulated drug more rapidly at the higher drug concentration.

Dicumarol had no effect on rapid drug uptake or accumulation in resistant 3437T cells under aerobic conditions (Fig. 2, C and D) but reduced the amount of drug accumulation in GM38 cells to that of 3437T at 0.625 μg/mL and close to 3437T levels at 7.0 μg/mL. Under hypoxic conditions (Fig. 2, dashed lines) DIC increased the rate of drug accumulation in 3437T cells at both PM concentrations and increased it markedly in GM38 cells at 0.625 μg/mL and slightly at 7.0 μg/mL. Loss of cell-associated radioactivity following the wash period was very similar under all conditions and did not demonstrate

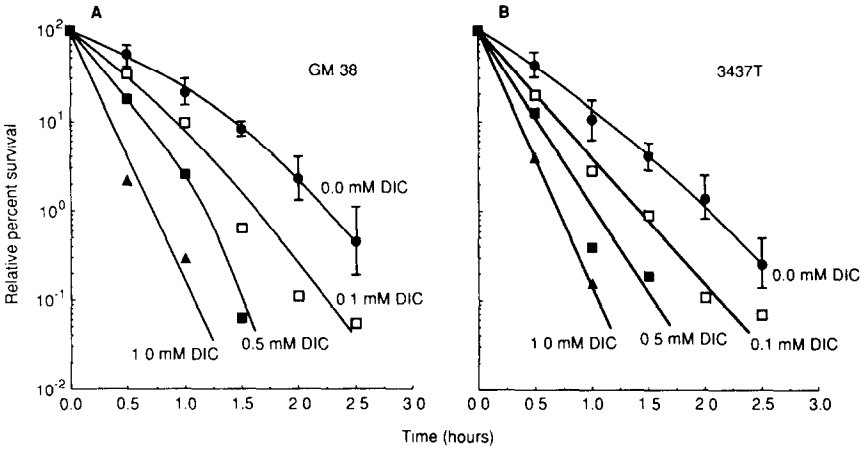

Fig. 3. Relative percent survival of GM38 and 3437T cells as a function of exposure time to 0.5 μg/mL PM under hypoxic conditions in the presence of different concentrations of DIC (single experiments). Results in the absence of DIC are the means of three experiments ± SD.

Table 1. GSH, DT-diaphorase and cytochrome P450 reductase activities of cell strains 3437T and GM38*

	GSH (μg/10^6 cells)	DT-diaphorase (nmol/min/mg protein)	Cytochrome P450 reductase (nmol/min/mg protein)
GM38	43 ± 10	1820 ± 200	6.6 ± 0.7
3437T	57 ± 14	30 ± 20	5.7 ± 0.3
P value	0.26	0.0048	0.21

* Mean values of at least three experiments ± SD are shown.

any trends which were not evident from the accumulation phase.

There was no difference in sensitivity to 0.5 μg/mL porfiromycin between the cell strains 3437T and GM38 under hypoxic exposure conditions (Fig. 3 and [14]). The effect of three concentrations of DIC on the toxicity of PM under hypoxic conditions was examined in three separate experiments. The results of exposure to PM in the absence of DIC for this series of experiments were pooled and are presented in Fig. 3 as means ± SD to indicate the degree of reproducibility between experiments. The addition of DIC during hypoxic exposures of either cell strain to PM increased toxicity, and the amount of cell killing increased as the DIC concentration increased from 0.1 to 1.0 mM. These DIC concentrations did not alter the plating efficiency of either cell strain in the absence of PM (data not shown). These results were reproduced qualitatively using another PM concentration (1.0 μg/mL) (data not shown).

Strain 3437T demonstrated a higher aerobic D_{10} than strain GM38 (16.5 ± 2.2 and 2.9 ± 0.5 μg/mL/hr, respectively, P = 0.001) and possessed negligible DT diaphorase activity (Table 1). Strain GM38 had DT-diaphorase activity approximately sixty times greater than that of strain 3437T. There was no inhibition of the DT-diaphorase activity when preparations of GM38 and 3437T were mixed and assayed together (data not shown). A small difference between the glutathione content of the two cell strains was noted, with levels being slightly higher in the 3437T cells. There were no significant differences in cell volume between the cell strains (3360 ± 360 vs 3370 ± 1320 μm^3 for GM38 and 3437T, respectively, P = 0.95), indicating that this result was not related to larger cell volume. Cytochrome P450 reductase activities were also very similar for both cell strains.

To determine if the reduced activity of DT-diaphorase in 3437T cells could be correlated with the amount of enzyme present in these cells, immunoprecipitation experiments were conducted. Proteins were precipitated from GM38 and 3437T cells with either a non-specific antibody (control) or a rabbit polyclonal antibody raised against a purified rat DT-diaphorase (αDTD) and then subjected to polyacrylamide gel electrophoresis (Fig. 4). After a 2-hr labelling period, a 32 kD protein was immunoprecipitated by DT-diaphorase antibody from GM38 cells but not 3437T cells. This molecular weight corresponds closely to that determined previously for DT-diaphorase isolated from human cells [33]. The virtual absence of DT-diaphorase activity and antibody-reactive protein in cell strain 3437T could not be accounted for by the absence of the DT-diaphorase gene. Southern analysis indicated that some form of a DT-diaphorase gene was present in both cell strains GM38 and 3437T (Fig. 5).

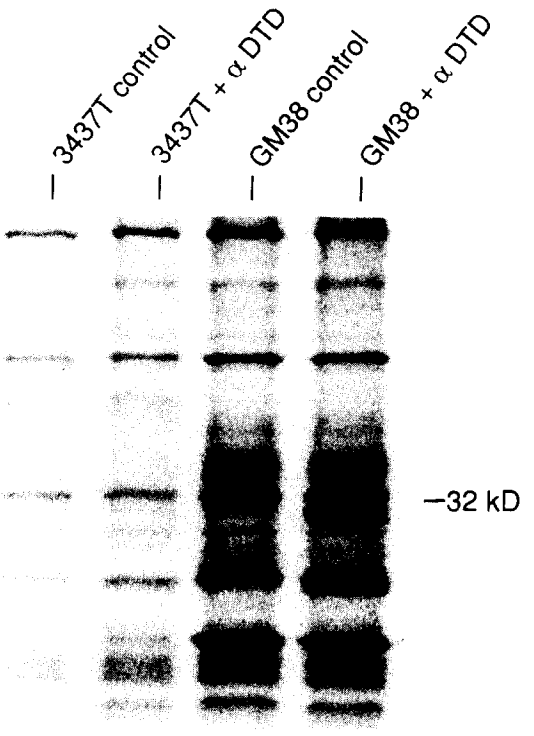

Fig. 4. Comparison of [^{35}S]methionine-labelled proteins isolated from cell strains GM38 and 3437T and immunoprecipitated with antibody against rat DT-diaphorase. Control lanes have been immunoprecipitated with non-specific human IgG to indicate DT-diaphorase-specific proteins in the corresponding lanes.

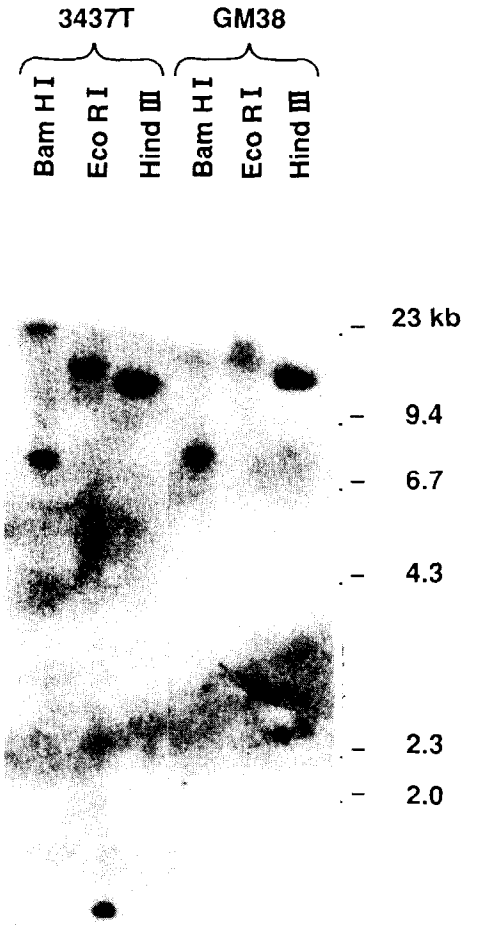

Fig. 5. Comparison of the fragments of genomic DNA obtained from GM38 and 3437T cells containing the DT-diaphorase gene. Genomic DNA has been digested to completion with the restriction enzymes indicated, transferred to nitrocellulose and probed with cloned fragments of the DT-diaphorase gene labelled with ^{32}P.

However, differences between the cell strains were noted with respect to the size of fragments generated with enzymes Bam HI and Eco RI.

DISCUSSION

Mitomycin C and PM may be activated to reactive forms by both one- and two-electron pathways. The relative role of the two pathways and/or the specific enzyme involved may be different for the two drugs. It was initially believed that two-electron reduction to the hydroquinone intermediate was required for cytotoxicity [34]. More recent evidence shows that one-electron reduction to the semiquinone intermediate is sufficient to generate active alkylating species [35]. Several enzymes have been studied for their potential role in these various pathways. NADPH–cytochrome *c* (cytochrome P450) reductase and xanthine oxidase may each reduce the parent compound by one-electron additions to produce sequentially the semi- and hydroquinone [36, 37]. The ability of oxygen to reoxidize these intermediates to the non-alkylating parent form would appear to account for the preferential hypoxic cytotoxicity of this class of compounds. However, some toxicity is observed in the presence of oxygen, suggesting that reduction to active species may still occur, although to a lesser extent than in the absence of oxygen, and/or that production of reactive oxygen species may contribute to cytotoxicity. The importance of these active oxygen species may be a function of the cell line and the specific mitomycin C analogue studied [6, 7]. An enzyme capable of direct two-electron reduction would bypass the more easily oxidizable semi-quinone step in the pathway activating these compounds to alkylating species while reducing the generation of reactive oxygen species. DT diaphorase is a cellular reductase which has been implicated in the aerobic metabolism of quinones such as menadione [38] and AZQ [39] and may also be involved in the aerobic cytotoxicity of MMC and PM [14].

To investigate the basis for aerobic resistance of cell strain 3437T, metabolic activation and generation of reactive alkylating species were monitored with an alkylation assay using PNBP as a trapping agent. This technique was used previously to demonstrate that the absence of oxygen leads to increased

bioreductive activation of MMC [17, 18]. In the present study it was again evident that generation of alkylating species was greater under hypoxic conditions for both resistant and normal cell strains with both MMC and PM (Fig. 1). However, generation of alkylating species could not be correlated in all cases with cytotoxicity. Although cell strain 3437T was resistant to MMC and PM under aerobic conditions, generation of alkylating species was similar for both cell strains. Conversely, while the cell strains were similar in PM and MMC sensitivity under hypoxic conditions (Fig. 3), differences were observed in their ability to produce alkylating species under hypoxic conditions (Fig. 1). Other discrepancies between survival data and the PNBP alkylation assay were also apparent. In this assay PM generated more alkylating species than MMC under aerobic conditions, while it has been demonstrated previously that PM is less toxic than equimolar concentrations of MMC under such conditions [40, 41]. Under hypoxic conditions, PM cytotoxicity was very similar to that of MMC in both cell strains [14], although PM was responsible for higher levels of alkylation than MMC under such conditions.

Discrepancies between cell survival data and the amount of alkylation observed may be due to several factors. Very high concentrations of drug (300 μM) are required to produce detectable levels of alkylation in this assay, whereas concentrations of 1.5 μM are sufficient to detect differences in cell survival assays. Also, the alkylation assay is quite non-specific in terms of the nature of the alkylating species which it will detect [42]. For example, generation of either the semiquinone or hydroquinone and monofunctional or bifunctional alkylating species could not be differentiated. Indeed, monofunctional alkylation is 10-fold more predominant than cross-link formation [43], suggesting that differences in bifunctional cross-linking potential may be masked in an assay which cannot differentiate between various alkylating species. Finally, the possible partitioning of activity in intracellular compartments may preclude a direct comparison of cell sonicate activities with results obtained using intact cells.

The total inability of the resistant cell strain, 3437T, to accumulate drug after the initial rapid uptake phase under aerobic conditions may reflect a deficiency in drug activation. The normal cell strain continually accumulated drug under aerobic conditions at low drug concentrations (Fig. 2A), but reached a plateau level within 30 min at the higher drug concentration (Fig. 2B), perhaps reflecting inactivation of drug-activating sites. Hypoxic conditions increased the rate of drug accumulation for both cell strains, consistent with such conditions either facilitating the activity of an oxygen-sensitive activation pathway or preventing reoxidation of reaction intermediates. The rate of accumulation under hypoxic conditions increased at the higher drug concentration, but there was no evidence of the plateau observed under aerobic conditions. These results agree with those reported previously by Keyes *et al.* [22] with EMT6 cells.

While the two cell strains used in the present experiments were similar in sensitivity to PM under hypoxic conditions, strain 3437T accumulated drug at a lower rate than GM38 under hypoxic conditions. Therefore, the rate of drug accumulation did not correlate quantitatively with hypoxic cell survival, suggesting that other factors, such as levels of cellular protective agents, rate of lesion repair or the nature of the damaging species themselves, may also contribute to differences in cell survival. The lack of any large difference in glutathione levels between the cell strains (Table 1) suggested that this intracellular agent was not involved in the resistant phenotype of the 3437T cells. The lack of glutathione involvement has also been noted by Dulhanty *et al.* [44] in CHO cell lines resistant to MMC. The similarity in cell volume indicated that differences in cell-associated radiolabelled drug and enzyme levels were not caused by variations in cell size between the strains.

The ability of DIC to decrease aerobic drug accumulation and cytotoxicity [14] in the normal but not the drug-resistant cell strain is consistent with this agent inhibiting a process of aerobic drug activation which is present in cell strain GM38 but not strain 3437T, probably mediated by DT-diaphorase. Conversely, under hypoxic conditions, DIC increased the rate of drug accumulation and increased PM cytotoxicity in both cell strains. Thus, the manner in which DIC alters drug accumulation and cytotoxicity depends upon the degree of oxygenation at the time of drug exposure. The nature of the effect of DIC on hypoxic toxicity of these compounds could also involve alterations in cellular respiration [45] or intracellular calcium homeostasis [38], areas that warrant further investigation.

Cell strain 3437T was found to be deficient in DT-diaphorase activity (Table 1). The absence of such an enzyme capable of reducing quinone-containing compounds under aerobic conditions could account for the inability of strain 3437T to accumulate the radiolabelled drug under aerobic conditions. The observation that DIC, a known inhibitor of DT-diaphorase, reduced accumulation of drug in the normal cell strain but not the drug-resistant cell strain provided further evidence that the absence of DT-diaphorase was related in some manner to the aerobic resistance to PM and MMC. It would appear, however, that the effect of DIC on PM toxicity under hypoxic exposure conditions is not dependent upon the presence of DT-diaphorase. This is in agreement with previously reported results of others [13, 46]. It can be argued that, under hypoxic conditions, the activity of other enzymes which activate drug by one-electron reduction steps overshadows the absence of the two-electron reduction pathway, leading to the similar sensitivities of the cell strains under hypoxic conditions.

Decreased DT-diaphorase activity was reflected in the absence of a 32 kD protein (Fig. 4) which corresponded closely with the molecular weight previously demonstrated for this enzyme in humans [33]. This could indicate that the rate of synthesis of DT-diaphorase is decreased significantly in strain 3437T, that a DT-diaphorase protein is produced but not detected in the immunoprecipitation experiment due to a decrease in its stability, or that

an altered DT-diaphorase protein is produced but not recognized by the rabbit-anti-rat DT-diaphorase antibody used in these experiments. Nonetheless, the absence of a protein band corresponding to the molecular weight of DT-diaphorase is consistent with the negligible activity of this enzyme observed in 3437T cells.

Southern analysis indicated that a DT-diaphorase gene was present in both the resistant and normal cell strains (Fig. 5). However, there were differences in the gene between the two cell strains as noted by the different fragment sizes produced by digestion with restriction enzymes Bam HI and Eco RI. This suggested that the genes differed structurally in some manner between normal and resistant cell strains. Jaiswal *et al.* detected no differences in the DT-diaphorase gene between ten individuals whose DNA was digested with eleven different restriction enzymes [33], suggesting that the restriction fragment length polymorphisms (RFLPs) noted in the present study were not reflections of RFLPs present in the general population. Further study of the structure and regulation of this gene in the MMC-resistant and normal cell strains will provide further information to explain the absence of DT-diaphorase activity in the drug-resistant cell strain.

The present results suggest that decreased DT-diaphorase activity is closely related to mitomycin resistance under aerobic conditions in human cell strain 3437T and may, in fact, be the cause of such resistance. Similar results have been reported recently for a series of CHO cell lines that were resistant to MMC and deficient in DT-diaphorase activity [44]. A murine lymphoblast cell line with increased levels of DT-diaphorase and increased sensitivity to MMC has also been described [47, 48]. DT-diaphorase activity has also been correlated with MMC sensitivity in two human colon carcinoma cell lines [49]. This body of evidence suggests that DT-diaphorase is involved in the metabolic activation of the quinone compounds MMC and PM, leading to the production of reactive intermediates which produce cytotoxic lesions. An alternate explanation that aerobic resistance is due to an enhanced ability of 3437T to deal with reactive oxygen species [6, 7] seems less likely on the basis of the current data but levels of catalase and superoxide dismutase and related enzymes have not been measured as yet.

Some controversy exists over the ability of MMC to act as a substrate for DT-diaphorase purified from various sources, with data supporting [50] and data arguing against [51] this activation pathway. A possible resolution to this controversy may be found in the recent observation that the ability of MMC to act as a substrate for DT-diaphorase is pH dependent [49]. It has also been shown that increased DT-diaphorase activity can occur in a coordinated fashion with increased expression of other enzymes such as cytochrome P450 reductase [52]. Indeed, resistance to MMC has been reported in a CHO cell line which demonstrated no DT-diaphorase activity and decreased NADPH:cytochrome P450 reductase activity [53]. However, the present data, and those of Begleiter *et al.* [47], indicate that altered DT-diaphorase activity need not always be associated with altered cytochrome P450 reductase activity in the human and murine cells studied. It is obvious, therefore, that isolation and purification from various sources of enzymes involved in mitomycin metabolism are crucial. An understanding of their structural genes and factors regulating their expression will establish a molecular basis for the activation of and cellular sensitivity to these compounds.

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