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MECHANISMS UNDERLYING RESISTANCE TO STREPTOZOTOCIN IN Mer$^+$ AND Mer$^-$ HUMAN TUMOR LINES

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Abstract—Streptozotocin (STZ) is a monofunctional nitrosourea employed in the treatment of patients with islet cell tumors. To analyze the role of DNA repair mechanisms in causing resistance to STZ, we evaluated the cytotoxicity by this agent in three human tumor lines that differ with respect to their abilities to repair *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) damaged virus (the Mer phenotype). HT-29, A2182, and BE human tumor lines are high, intermediate and low, respectively, with regard to features that define the Mer phenotype. Our results demonstrated that the order of resistance to STZ is HT-29 > A2182 > BE. The degree of inhibition of DNA synthesis by STZ was in the following order: BE > A2182 > HT-29. O^6-Alkyltransferase activity was increased markedly in HT-29 cells compared to A2182 cells which, in turn, had significantly increased levels compared to the BE line. Other potential factors such as 3-methyladenine DNA glycosylase activity, the induction by STZ of single-stranded DNA breaks, and the kinetics of repair of these breaks do not clearly underlie differences in cytotoxicity among the three tumor lines. However, increased topoisomerase II activity, as well as enhanced sensitivity to agents that interact with topoisomerase II, was present in A2182 cells compared to BE cells. These findings demonstrate that while O^6-alkyltransferase contributes to resistance to STZ in some Mer$^+$ tumor lines, other mechanisms may also contribute to resistance to this agent.

Streptozotocin (STZ) is a monofunctional nitrosourea employed in the treatment of patients with insulinoma and carcinoid tumors [1, 2]. This agent causes diabetes in animals [3, 4] and is also a potent mutagen and carcinogen [5–7].

Earlier studies suggested that STZ causes cytotoxic effects in eukaryotic cells by inducing DNA strand scission, thus activating poly ADP ribose synthetase and lowering levels of NAD and ATP within the cell [8]. Whether other mechanisms affect the cytotoxicity by this agent in human tumors, however, is unclear.

Our prior work examined the role of DNA repair mechanisms in affecting cytotoxicity by STZ in *Escherichia coli*. The adaptive response in *E. coli* is induced by STZ [9]. This repair mechanism prevents both mutagenic and cytotoxic effects by methylating and ethylating agents [10, 11]. Both the adaptive response as well as recombinational repair are critical in ameliorating toxicity by STZ since *E. coli* mutants deficient in these repair pathways were highly sensitive to STZ.† Mutants lacking 3-methyladenine DNA glycolase activity are particularly sensitive, suggesting that this DNA base adduct is an important cytotoxic lesion in *E. coli* [6].

In the studies described in this report, we analyze mechanisms underlying cytotoxicity by STZ in human tumor lines that are highly proficient, intermediate, or deficient in their capacity to repair *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) damaged virus. The Mer repair phenotype is defined by the ability of the cell to repair alkylator-damaged virus and is attributed to increased levels of O^6-alkyltransferase activity [12, 13]. We endeavored to evaluate whether 3-methyladenine DNA glycosylase activity in addition to O^6-alkylguanine-DNA alkyltransferase affected survival after treatment with STZ as was the case in *E. coli*. Since increased activity of topoisomerase II, an enzyme involved in mediating topological rearrangements in DNA, was implicated in causing resistance to an alkylating agent (nitrogen mustard) in a human tumor line [14], we also measured the activity of this enzyme.

METHODS

Human tumor lines

HT-29, A2182, and BE tumor lines were obtained from Dr. L. Erickson (Loyola University Medical Center). These lines were derived originally from human colon (HT-24 and BE) and lung (A2182) carcinomas and were evaluated previously for their abilities to enhance survival of MNNG damaged virus. While both HT-29 and A2182 tumor lines are Mer$^+$, the BE line is Mer$^-$ [12, 13]. Cells were grown in Eagle's Minimum Essential Medium with 10% fetal calf serum, L-glutamine (0.03 μg/mL), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM), D-biotin (0.1 μg/mL), B12 (1.36 μg/mL), penicillin/streptomycin (1000 units/mL and 100 μg/mL respectively), and gentamicin (0.05 mg/mL).

Cytotoxicity

Cells (250, 500, 1000, 5000, 10^4, and 5×10^4) were

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† Fram RJ, Mack SL and Marinus MG, unpublished data.

plated onto 100 mm plastic dishes (Corning, Corning, NY) and allowed to attach overnight. Cells were then exposed to drug for 1 hr at 37°, washed twice, and incubated in a 5% CO_2 environment for 10–14 days [15]. Colonies were then stained and counted as previously described. Plating efficiency was 20, 20 and 17% for HT-29, A2182, and BE cell lines respectively. Percent control survival was calculated by dividing the numbers of colonies obtained in drug-treated cells by those found in cells not exposed to drug and multiplying by 100. Results are the means of two independent experiments each performed in triplicate. Standard deviations were less than 5% of the mean. Cells were in exponential growth phase during all experiments.

Reagents

Streptozotocin was obtained from the Sigma Chemical Co. (St Louis, MO) and was dissolved in medium. Teniposide and etoposide were gifts from the Bristol Myers Corp. (Evansville, IN), and a 0.1 M stock solution of drug dissolved in dimethyl sulfoxide (DMSO) was prepared just prior to use. The final concentration of DMSO was less than 0.5% and was not toxic. 4′-(9-Acridinylamino)-methanesulfon-*m*-anisidide (m-AMSA) was provided by the Drug Synthesis and Chemistry Branch (DTP, DCT, NCI) and was dissolved in medium.

O^6-Alkylguanine DNA alkyltransferase activity

O^6-Methylguanine [^{3}H]DNA was prepared by exposing 5 mg *E. coli* DNA (Sigma) to [^{3}H]*N*-methyl-*N*-nitrosourea ([^{3}H]MNU) (1 Ci/mmol; New England Nuclear, Boston, MA) according to a previously published procedure [16]. The specific activity was 40,000 cpm/mg DNA. About 10^7 cells were trypsinized, spun, and washed with phosphate-balanced salt solution (PBS). The pellet was lysed with 0.3 M NaCl, 0.05 M Tris–HCl, 10 mM EDTA, 1 mM dithiothreitol (pH 8.0) and sonicated for 5 min. Cell debris was removed by centrifuging the solution for 5 min at 15,000 rpm. The supernatant fraction was removed, and protein was measured by a modification of the Lowry assay [17] using a protein assay kit (Sigma).

A 350 μL sample of cell extract (875–3500 μg protein) was added to 50 μL (10×) buffer, 100 μL [^{3}H]DNA (80 μg DNA), and double-distilled water to a volume of 500 μL with a final concentration of 70 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes)-KOH, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, pH 7.8. Bovine serum albumin (BSA) was added so that all samples had 3500 μg protein. Controls consisted of 3500 μg BSA without cell extract. The solution was incubated 1 hr at 37°, cooled to 0°, and then DNA and protein were precipitated with 0.5 mL of cold 0.8 M trichloroacetic acid (TCA). The solution was centrifuged at 2500 rpm for 20 min at 0° and decanted. The pellet was washed with 1 mL of cold (−20°) 95% ethanol and spun at 2500 rpm at 4° for 20 min. DNA was hydrolyzed by adding 2 mL of 0.1 N HCl and then incubated for 1 hr at 70°. The solution was spun as previously described. The supernatant fraction was removed and spun at 1500 rpm for 10 min through a 0.45 μm nylon filter (Alltech, Deerfield, IL).

The presence of tritiated radioactivity in [^{3}H]O^6-methylguanine was confirmed by HPLC. O^6-Methylguanine was prepared from O^6-methylguanosine (provided by Dr. D. Ludlum, Department of Pharmacology, University of Massachusetts Medical School) by dissolving the latter in 0.1 N HCl and heating at 70° for 1 hr. The HPLC system employed an HS C18 column (Alltech) as well as a C18 prefilter (Waters, Milford, MA). The mobile phase consisted of 10% methanol and 90% 250 μM potassium phosphate. Absorbance was analyzed at a wavelength of 254 nm. The pump flow rate was 1 mL/min. Radioactivity eluting in the same fractions as an O^6-methylguanine standard was measured.

Tritiated O^6-methylguanine removed from the DNA was calculated by determining radioactivity in DNA when cellular protein was not added and subtracting values obtained after the addition of various amounts of cellular protein during the initial incubation with [^{3}H]DNA.

Measurement of 3-methyladenine DNA glycosylase activity

Preparation of DNA substrates. [^{3}H]3-Methyladenine in DNA was formed by incubating calf thymus DNA (Sigma Type 1) with [*methyl*-^{3}H]-dimethylsulfate (2.14 Ci/mmol; New England Nuclear), using a previously published method [18]. The specific activity was 3.1×10^4 dpm/μg DNA.

3-*Methyladenine DNA glycosylase activity.* Protein (0–100 μg) from cell extract (prepared as previously described) was added to 2.5 μg [^{3}H]DNA in a final volume of 200 μL buffer (the same as employed in the prior assay except 5% glycerol also was added). The solution was incubated at 37° for 60 min and then placed on ice for 10 min. One hundred microliters of heat-denatured calf thymus DNA (2 mg/mL in 1 M NaCl) and 500 μL of cold absolute alcohol were added. The solution was kept at −20° overnight and then centrifuged at 25,000 rpm for 30 min at 0°. The supernatant fraction was decanted into 5 mL of Optifluor (Packard, Downer Groves, IL). Radioactivity was then measured with a scintillation counter. Results are expressed as picomoles of 3-methyladenine removed from DNA. Aliquots of the supernatant fraction also were evaluated by HPLC. Ethanol was removed from samples under a stream of nitrogen gas and redissolved in 200 μL of double-distilled H_2O with standards for 3-methyladenine, 3-methylguanine, and 7-methylguanine (Sigma). Debris was removed by centrifugation through a 0.45 μm nylon filter (Alltech). Bases were separated using the same HPLC system employed for measuring O^6-methylguanine (see above). Results demonstrated that 75% of the radioactivity consisted of 3-methyladenine and 25% of 7-methylguanine.

Inhibition of DNA synthesis

To measure inhibition of DNA synthesis, 2×10^6 cells were allowed to settle on 25 cm flasks overnight. Next 5 μCi/mL [*methyl*-^{3}H]thymidine (sp. act. 2 Ci/mmol; New England Nuclear) was added with either no drug or 10^{-4}–5×10^{-3} M STZ for 1 hr at 37°. Cells were washed, trypsinized, centrifuged (1500 rpm for 3 min), and then resuspended in 1 mL of 10 mM Tris, 1 mM EDTA, 5% SDS and 0.5 mg/mL proteinase

K and left overnight at 37°. Cold 5 M NaCl (1/10 vol.) and absolute ethanol (2× vol.) at −20° were added, and the solution was placed at −20° for 2 hr. DNA was isolated by centrifugation and resuspended in 1 mL of 20 mM Tris and 1 mM EDTA; 2 ml of 25% cold TCA was added, and the solution was placed on ice for 30 min. The samples were then filtered on Whatman GF/A filters (Whatman, UK), and radioactivity was analyzed after the addition of 5 mL Opti-fluor.

Alkaline elution

The procedure for alkaline elution analysis has been published elsewhere [19]. HT-29, BE, and A2182 cells were labeled with 0.02 μCi/mL [^{14}C-*methyl*]thymidine (53.2 mCi/mmol; New England Nuclear) for 24 hr, washed, and treated with no drug or STZ for 1 hr. Cells were then washed, trypsinized, and resuspended in cold PBS. Internal standard cells consisted of cells from each of the respective tumor lines that were labeled with 0.1 μCi/mL [*methyl*-^{3}H]thymidine (sp. act. 2 Ci/mmol; New England Nuclear) for 24 hr, washed, trypsinized and resuspended in cold PBS. Cells were irradiated with 150 R. Cells (5×10^5 for drug treated and for internal standard) were added to 2 μm polycarbonate filter (Nucleopore Co., Pleasanton, CA) in 10 mL of cold PBS. The solution was allowed to drain by gravity through the filters. Cells were washed with an additional 10 mL of cold PBS. Cells were lysed with 5 ml of 2% sodium dodecyl sulfate (SDS), 0.25 M NaEDTA, pH 9.7, followed by 2 mL of the same solution with proteinase K (0.5 mg/mL). Tetrapropylammonium hydroxide (pH 12.1) with 0.1% SDS and 0.2 M EDTA was pumped at a rate of 0.04 mL/min over the filters. Fractions were collected at 3-hr intervals over 15 hr and assayed for radioactivity after the addition of 14 mL scintillation fluid.

Measurement of topoisomerase II activity

Isolation of [^{3}H]*kinetoplast DNA. Crithidia fasciculata* was a gift from Dr. B. Glisson (M.D. Anderson Hospital and Tumor Institute). Cells (2×10^4) were grown in plastic flasks (250 mL) with 42 mL brain heart medium and 0.42 mL hemin at room temperature until a density of 4–5 × 10^7 cells/mL was reached. The contents of three flasks were added to 174 mL/medium and shaken in a water bath at 27°. After 3 hr, 1.0 mCi [^{3}H]thymidine (20 Ci/mmol, New England Nuclear) was added and the cells were labeled for 1 hr. Cells were shaken for 24 hr until a density of 1–1.5 × 10^8/mL was reached. They were then spun at 6000 rpm for 10 min and resuspended in 15 mL of cold 100 mM NaCl, 10 mM Tris, 100 mM EDTA. A cell lysate was produced after addition of 15 mL of 6% Sarkosyl with 50 μg/mL ribonuclease and 500 mg/mL proteinase K at 37° and incubated at 4° for 1 hr. Cell lysate was added to CsCl (final density was 1.72 g/mL) with 300 μg/mL ethidium bromide and spun for 72 hr at 37,000 rpm at 20° on a Beckman Ti60 rotor. Lower DNA bands were collected by side puncture and extracted three times with isopropranol. DNA was dialyzed against 200 vol. 1 mM Tris, 10 mM EDTA at 4° overnight.

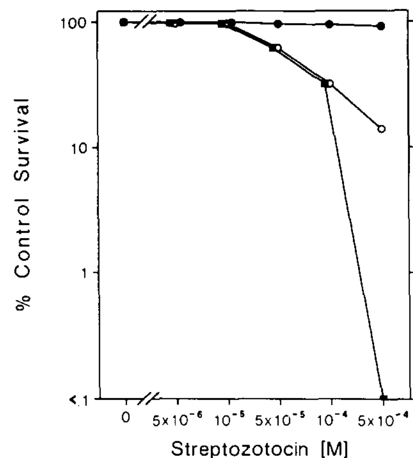

Fig. 1. Cytotoxicity by streptozotocin in HT-29, A2182 and BE cells. Cells were exposed to STZ for 1 hr at 37°, washed, trypsinized and plated and then incubated in a 5% CO_2 environment, 37°, for 10–14 days. Colonies of more than 50 cells were counted. Results are the means of two independent experiments with each determination performed in triplicate. Standard deviations were less than 5% of the means. Percent control cell survival was calculated as described in Methods. Key: HT-29 (●), A2182 (○), and BE (■) cells.

DNA was precipitated with 3 vol. cold (−20°) absolute ethanol. DNA was spun on a glass rod and resuspended in buffer. The presence of kinetoplast DNA (k-DNA) was verified by its failure to migrate in a 1% agarose gel. The specific activity of the k-DNA was 2717 cpm/μg DNA.

Assay of topoisomerase II activity. This technique which monitors the decatenation of tritiated k-DNA after the addition of a cell lysate in the presence of ATP has been published previously [20]. The assay is specific and quanitative for topoisomerase II activity, and prior results have correlated with assays that analyze decatenation by measuring migration in agarose gels. Protein determinations on cell extracts were performed with a Bio-Rad protein assay (Bio-Rad, Richmond, CA). Results are expressed as ng k-DNA released.

RESULTS

Cytotoxicity in Mer^{+} and Mer^{-} human tumor lines by STZ is shown in Fig. 1. Mer^{-} BE cells were markedly more sensitive than Mer^{+} A2182 cells. The latter were, in turn, more sensitive than HT-29 cells.

To explore mechanisms underlying the cytotoxicity by STZ in these tumor lines, the extent of inhibition of DNA synthesis by STZ was analyzed. Figure 2 demonstrates that, as with cytotoxicity, DNA synthesis was inhibited in a rank order corresponding to the cytotoxicity results (BE > A2182 > > HT-29). While the extent of inhibition of DNA synthesis varied among the three tumor lines, no differences in either the induction of DNA single-strand breaks or the repair of these breaks were present, as analyzed by alkaline elution (data

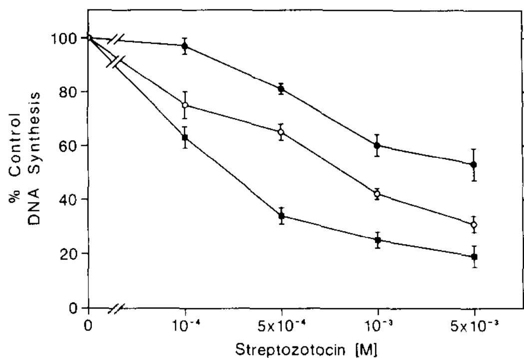

Fig. 2. Inhibition of DNA synthesis by STZ in HT-29, A2182, and BE cells. Cells were treated with 5 μCi/mL [^{3}H]thymidine either with 10^{-4}–5×10^{-3} M STZ or no drug for 1 hr at 37°. Cells were washed and trypsinized, and DNA was isolated. Key: HT-29 (●), A2182 (○), and BE (■) cells. Values are the means ± SD of two independent experiments performed in duplicate.

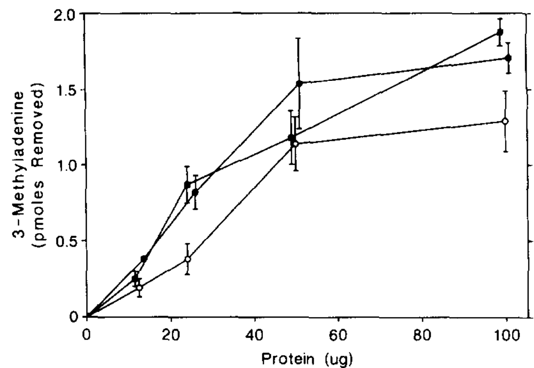

Fig. 4. 3-Methyladenine DNA glycosylase activity in HT-29, A2182, and BE cells. Removal of 3-methyladenine was analyzed by measuring the release of radioactivity from [^{3}H]dimethylsulfate-treated DNA after exposure to cellular extract. Values are the means ± SD of two independent experiment, performed in duplicate. See Methods for details. Key: HT-29 (●), A2182 (○), and BE (■) cells.

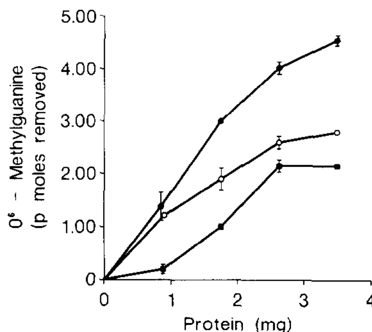

Fig. 3. O^6-Alkylguanine-DNA-alkyltransferase activity in HT-29, A2182 and BE cells. Cells (10^7) were trypsinized, spun, washed and lysed. O^6-Alkylguanine-DNA-alkyltransferase activity was analyzed by assessing the removal of O^6-methylguanine from [^{3}H]MNU-treated DNA. Results are the means ± SD of two determinations from a representative experiment. Key: HT-29 (●), A2182 (○), and BE (■) cells.

not shown). Since O^6-alkylguanine-DNA-alkyltransferase activity is implicated in mediating resistance to alkylating agents in Mer$^+$ tumor lines [21], this activity was analyzed in the three tumor lines. As shown in Fig. 3, O^6-alkyguanine-DNA-alkyltransferase activity was increased markedly in HT-29 cells compared to A2182 cells. Further, the A2182 tumor line also had markedly greater alkyltransferase activity than the BE line.

Since our prior work suggested a role for 3-methyladenine DNA glycosylase in improving survival after treatment of *E. coli* cells with STZ [6], we analyzed this repair enzyme activity in the three human tumor lines. The results (see Fig. 4) demonstrated that levels of 3-methyladenine DNA glycosylase activity are not significantly different between HT-29 and BE cells and, therefore, do not explain the differences in survival among the tumor lines after STZ treatment.

Topoisomerase II activity also was analyzed since this enzyme activity was implicated recently in mediating resistance to the alkylating agent mechlorethamine [14]. The initial approach consisted of analyzing cytotoxicity in the three tumor lines by agents that bind to topoisomerase II. Cytotoxicity of this class of agents is often inversely correlated with levels of topoisomerase II in the absence of other factors such as differences in transport of drug [14]. Figure 5 demonstrates that among the tumor lines, A2182 cells were most sensitive to etoposide (VP-16), teniposide, and m-AMSA.

A more direct analysis of topoisomerase II activity is shown in Fig. 6. This enzyme specifically mediates decatenation of k-DNA in the presence of ATP [20]. The results show that markedly increased topoisomerase II activity was present in A2182 cells in comparison to either BE or HT-29 cells.

DISCUSSION

Our findings demonstrate that increased O^6-alkylguanine-DNA-alkyltransferase activity may underlie enhanced survival by Mer$^+$ HT-29 cells after exposure to STZ. These results are in keeping with the previously reported resistance of HT-29 cells to bifunctional alkylators as well as methylating agents [13]. That neither 3-methyladenine DNA glycosylase nor topoisomerase II activity was increased in HT-29 cells compared to the other tumor lines emphasizes the importance of this DNA repair mechanism in mediating resistance to STZ. Our results are consistent with the hypothesis that O^6-methylguanine is a cytotoxic lesion that interferes with DNA synthesis in human cells.

A2182 lung carcinoma cells were classified previously as Mer$^+$ on the basis of their ability to support the growth of nitrosoguanidine damaged adenovirus. This tumor line is intermediate in its sensitivity to MNNG compared to other Mer$^+$ and Mer$^-$ tumor lines as analyzed by clonogenic survival [12, 13]. In contrast, BE colon carcinoma cells are highly sensitive to a variety of methylating and chloro-

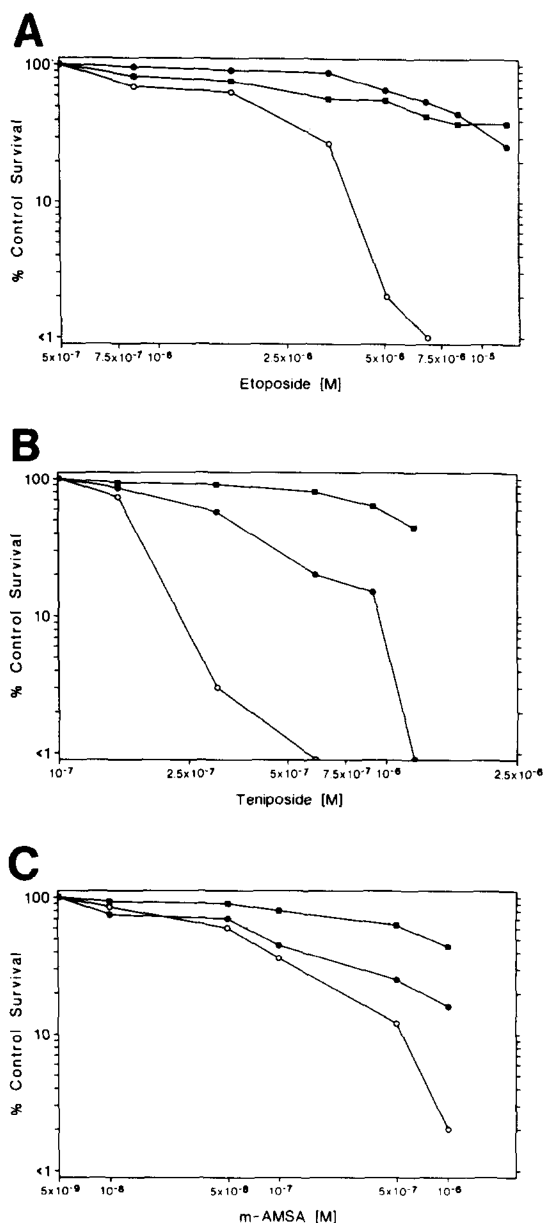

Fig. 5. Cytotoxicity by etoposide (A), teniposide (B), and m-AMSA (C) in HT-29, A2182 and BE cells. Cells were exposed to drug for 1 hr at 37°, washed, trypsinized, and plated. Cells were incubated for 14 days at 37°, 5% CO_2. Colonies with more than 50 cells were counted. Values are the means of two independent experiments performed in duplicate. Standard deviations were less than 5% of the mean. Key: HT-29 (●), A2182 (○) and BE (■) cells.

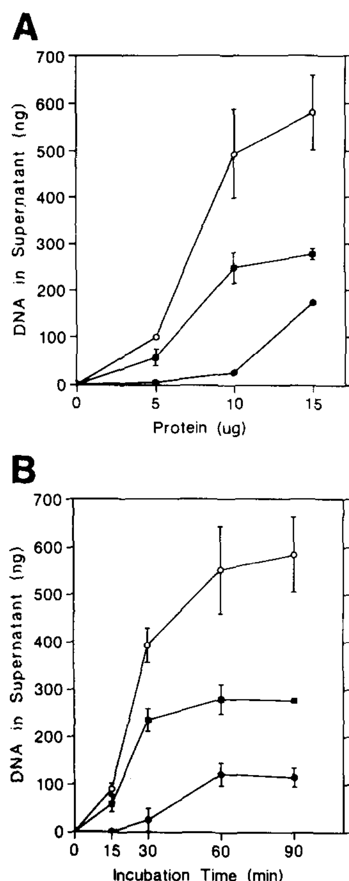

Fig. 6. Topoisomerase II activity in HT-29, A2182, and BE cells. The decatenation of tritiated k-DNA was analyzed in the presence of cellular extract and ATP. The effects of various protein concentrations of the cell extract (A) and duration of incubation after the addition of 15 μg protein from cell extracts made from the three tumor lines (B) were assessed. Values are the means ± SD of two independent experiments performed in duplicate. Key: HT-29 (●), A2182 (○) and BE (■) cells. See Methods for details.

ethylating agents that bind at the O^6 position of guanine [12].

Increased O^6-alkyltransferase activity underlies, at least in part, enhanced survival of A2182 cells compared to Mer$^-$ BE cells after exposure to STZ. While a prior report demonstrated no evidence of O^6-methyltransferase activity in BE cells [22], our results revealed that although levels are low, some O^6-methylguanine is removed by the BE cellular extract. Such variability in results likely occurs from differences in the techniques and cells employed.

Our results also demonstrated that topoisomerase II activity is increased in the A2182 tumor line compared to the BE line. These findings raise the issue of whether topoisomerase II may contribute in causing resistance to STZ. Our results, however, do not definitively document such a role. Topoisomerase II is closely associated with DNA and causes double-stranded nicks in DNA, thus permitting topological changes in this macromolecule so as to permit DNA replication and the segregation of chromosomes [23, 24]. This enzyme also is implicated in mediating resistance to the bifunctional alkylating agent mechlorethamine. While mechlorethamine-resistant squamous cancer cells form fewer DNA interstrand cross-links after drug treatment compared to a sensitive parent line, similar kinetics of cross-link excision as well as drug transport were noted [14]. These findings were consistent with enhanced mono-adduct repair resulting from increased topoisomerase II activity, the latter perhaps affecting

chromatin structure. Other factors thay may underlie differences in cytotoxicity by STZ in the three tumor lines include alterations in drug transport and/or drug inactivation. The formation of equal numbers of single-stranded DNA breaks after exposure to various concentrations of STZ among the three tumor lines largely excludes these possibilities.

The lack of any clear role for 3-methyladenine DNA glycosylase in mediating resistance to STZ contrasts with our prior observations in *E. coli alkA* mutants deficient in this repair enzyme [6]. These mutants are highly sensitive to STZ [6]. Our findings suggest that fundamental differences likely exist in the role of this repair mechanism in human tumor cells compared to prokaryotes. Our results also imply but do not prove that factors other than alkyltransferase may contribute to resistance to STZ. Studies are underway to determine whether mechanisms analyzed in the current study are relevant to Mer$^-$ cells made resistant to STZ.

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