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DNA Damage and Cytotoxicity of 2-Chloroethyl (Methylsulfonyl)methanesulfonate (NSC 338947) Produced in Human Colon Carcinoma Cells with or without Methylating Agent Pretreatment

John A. Hartley and Neil W. Gibson[1]

Laboratory of Molecular Pharmacology, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Maryland 20205

ABSTRACT

2-Chloroethyl (methylsulfonyl)methanesulfonate (ClEtSoSo) was more toxic to the BE (Mer⁻) cell line than to the HT-29 (Mer⁺) colon carcinoma. The sensitivity of the BE cells closely paralleled the induction of DNA interstrand cross-links by ClEtSoSo. No DNA interstrand cross-link formation was detected in the HT-29 cells after exposure to ClEtSoSo. Pretreatment of the HT-29 cells with methylating agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or streptozotocin increases their sensitivity to ClEtSoSo. Little or no increase in the toxicity of ClEtSoSo was found in BE cells after methylating agent pretreatment. Despite the increase in cell killing, no DNA interstrand cross-links were induced by ClEtSoSo after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine pretreatment. In contrast, streptozotocin pretreatment allowed ClEtSoSo to form DNA interstrand cross-links in HT-29 cells. The production of DNA strand breaks by ClEtSoSo was observed in HT-29 cells both with and without methylating agent pretreatment. These results suggest that the mechanism of ClEtSoSo may differ from other chloroethylating agents such as the chloroethylnitrosoureas. In addition, there may be a difference in the mechanism by which streptozotocin or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine pretreatment causes an increased cell killing in a previously resistant human colon carcinoma cell line.

INTRODUCTION

ClEtSoSo[2] is a newly synthesized compound which demonstrates curative action against a variety of murine tumors (1, 2). Tests in the National Cancer Institute tumor system showed that ClEtSoSo is as good as the ClEtNus, and the compound is being developed for clinical trials (1, 2). We have recently showed that, similarly to the ClEtNus, ClEtSoSo produced delayed interstrand cross-linking in guanine O^6-alkyltransferase deficient (Mer⁻) human tumor cell lines, but not in the proficient Mer⁺ cells (3). In addition, ClEtSoSo was shown to be much more selective in its alkylation of DNA, producing only chloroethyl adducts, with no hydroxyethyl adducts which were the major product with a ClEtNu (4). In a parallel study the base sequence selectivity for the reaction of these compounds at guanine N^7 positions of DNA was shown to be different (5).

Alkylation of the O^6-position of guanine has been suggested to be important in determining cellular sensitivity to chloroethylating agents (6). In particular, human tumor cells which are capable of repairing alkylations at the O^6 position of guanine are more resistant to the cytotoxic effects of chloroethylating agents than those without this repair activity (3, 6, 7). Such cells are described as possessing either the Mer⁺ phenotype or the Mer⁻ phenotype (8). The HT-29 cell line is proficient in the repair of guanine O^6 alkylation (Mer⁺), whereas the BE cell line is deficient in the repair of guanine O^6 alkylation (Mer⁻) (8).

It has recently been shown that pretreatment of Mer⁺ cells with the methylating agent MNNG inactivates the enzymatic activity responsible for the removal of O^6-alkylguanine lesions and thus allows the formation of DNA interstrand cross-links by agents such as ClEtNus (9), and mitozolomide (10). In addition, this pretreatment was found to greatly enhance the killing of Mer⁺ cells by both agents (9, 10). Pretreatment of Mer⁻ cells, however, had little effect on either cell killing or induction of DNA interstrand cross-links (9, 10). Similarly, pretreatment with the clinically active, and less cytotoxic, methylating agent STZ produced DNA interstrand cross-linking by both ClEtNus[3] and mitozolomide[4] in Mer⁺ cells. These data agree with the hypothesis that chloroethylating agent-induced cross-links are produced in DNA via chloroethyl monoadduct formation at the O^6 position of guanine, followed by the completion of an interstrand cross-link in a delayed reaction with the opposite DNA strand (11). The formation of such cross-links by the ClEtNus has been prevented by a guanine O^6-alkyltransferase activity purified from an *Escherichia coli* extract (12). Furthermore, Brent (13) has recently showed that DNA cross-link formation, induced by the ClEtNus, is inhibited by an extract from cultured human leukemic lymphoblasts (13). This activity, which inhibits cross-link formation, copurifies with O^6-methylguanine DNA methyltransferase and shows similar kinetic properties (13). Thus the same enzyme may be responsible for the removal of both methyl and chloroethyl monoadducts from the O^6 position of guanine.

In the present study we have examined the effects of methylating agent pretreatment on the cytotoxicity and DNA interstrand cross-linking of the novel agent ClEtSoSo in two human colon carcinoma cell lines, one determined to be Mer⁺ (HT-29) and the other Mer⁻ (BE) (14).

MATERIALS AND METHODS

Cell Culture. BE colon carcinoma cells were obtained from Dr. B. Giovanella, St. Joseph's Hospital Cancer Research Laboratory, Houston, TX. HT-29 colon carcinoma cells were obtained from Dr. E. Jenson, Mason Research Institute, Rockville, MD. Both cell lines have been maintained in this laboratory for several years. Stock cell cultures were grown at 37°C as monolayers in 75-cm² tissue culture flasks in Eagle's minimal essential medium (Dutchland Laboratories, Denver, PA) supplemented with 10% fetal bovine serum, gentamicin (0.05 mg/ml), glutamine (0.3 mg/ml), D-biotin (0.1 μg/ml), vitamin B_{12} (1.36 μg/ml), 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

For DNA alkaline elution studies, 2.5×10^5 cells were seeded into 25-cm² flasks in 10 ml Eagle's minimal essential medium and labeled for 24 h with [^{14}C]thymidine (0.02 μCi/ml; New England Nuclear; specific activity, 52 mCi/mmol). The labeling period was followed by

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[1] Present address: Department of Pharmacology, Fox Chase Cancer Center, Central and Shelmire Avenues, Philadelphia, PA 19111. To whom requests for reprints should be addressed.

[2] The abbreviations used are: ClEtSoSo, 2-chloroethyl (methylsulfonyl)-methanesulfonate; ClEtNus, chloroethylnitrosoureas; STZ, streptozotocin; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

[3] L. C. Erickson, personal communication.

[4] N. W. Gibson, J. A. Hartley, D. Barnes, and L. C. Erickson, unpublished observations.

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an 18-h incubation in fresh medium to allow for the incorporation of labeled DNA into high molecular weight DNA.

L1210 mouse leukemia cells were grown in suspension culture in RPMI 1640 medium supplemented with 15% heat inactivated (60°C, 45 min) fetal bovine serum. The DNA of L1210 cells was labeled by growing 3×10^5 cells/ml for 20 h in medium supplemented with [^{3}H]-thymidine (0.05 μCi/ml; New England Nuclear; specific activity, 20 Ci/mmol) and 10^{-6} M unlabeled thymidine.

Drug Treatment. MNNG was obtained from Aldrich Chemical Company, Milwaukee, WI. Drug was dissolved in 95% ethanol and stored at −20°C as 1000× stock at 0.001 M. STZ and ClEtSoSo were obtained from the Drug Development Branch, National Cancer Institute. STZ was dissolved in 0.02 M sodium citrate (pH 4.4) prior to use. ClEtSoSo was dissolved in sterile dimethyl sulfoxide immediately before treatment of cell cultures. The concentration of dimethyl sulfoxide in either treated or control cells was never greater than 2% (v/v). MNNG and STZ were added to cultures for 1 h at 37°C; this medium was then removed before the addition of ClEtSoSo for an additional 2 h. Treatments were terminated by aspiration of the drug containing medium and replacement with fresh Eagle's minimal essential medium.

Colony Forming Assays. HT-29 and BE cells were seeded at 0.1, 0.3, 1, 3, and 10×10^3 cells/25-cm² plastic flask (Costar, Cambridge, MA). The flasks were incubated for 12–20 h to allow the cells to attach to the bottom of the flask. Pretreatment of BE cells with 0.2 μM MNNG and HT-29 cells with 2 μM MNNG or 2.5 mM STZ was for 1 h where appropriate, followed by a medium change, and ClEtSoSo was added for an additional 2 h. After 10 days of incubation in fresh media, the flasks were rinsed with Hanks; balanced salt solution, fixed with methanol, and then stained with a solution containing 1 ml methylene blue, 1 ml 0.15 M Na_2HPO_4, and 1 ml 0.15 M KH_2PO_4 diluted to 50 ml with distilled water. Colonies were counted and the observed plating efficiencies were approximately 65% for HT-29 cells and 40% for BE cells.

Assay of DNA Damage by Alkaline Elution. The basic principles involved in the detection of DNA damage by the alkaline elution assay have been published and the methodology has recently been reviewed in detail (15). In order to accommodate the quantity of MNNG induced single strand breaks the modification of Zlotogorski and Erickson (9) was initially followed. Cells that were pretreated with MNNG were only irradiated with 150 rads of γ-rays. This was done because 2 μM MNNG was found to produce a quantity of DNA strand breaks similar to that caused by 150 rads of γ-rays (data not shown). The MNNG pretreatment plus 150 rads γ-ray exposure produced the same effect as 300 rads γ-ray exposure to cells that were not treated with MNNG.

For fast elution studies the DNA was eluted at 6 ml/h and five 1-h fractions were collected.[4]

RESULTS

Assays of the cytotoxicity of ClEtSoSo to HT-29 and BE colon carcinoma cells are shown in Fig. 1. These results are in agreement with the Mer status of the cells, BE (Mer⁻) cells being more susceptible to the drug. When the cells are pretreated with the methylating agent MNNG there is a moderate increase in BE cell killing; however, in the HT-29 cells a large increase in cell killing is observed relative to ClEtSoSo alone. These results are in agreement with those published previously for the ClEtSoSo (9) and another chloroethylating agent, mitozolomide (10).

The appearance of DNA-DNA interstrand cross-links was examined in both cell lines treated with ClEtSoSo with or without the pretreatment step with MNNG (Fig. 2). ClEtSoSo induced DNA interstrand cross-linking in the BE cell line and similar levels of cross-linking were observed in these cells following pretreatment. In the HT-29 cell line ClEtSoSo did not produce any DNA interstrand cross-links and, surprisingly, pretreatment of these cells with 2 μM MNNG failed to induce cross-links. This is contrary to previous studies with these cell lines where the increased cell killing observed in the HT-29

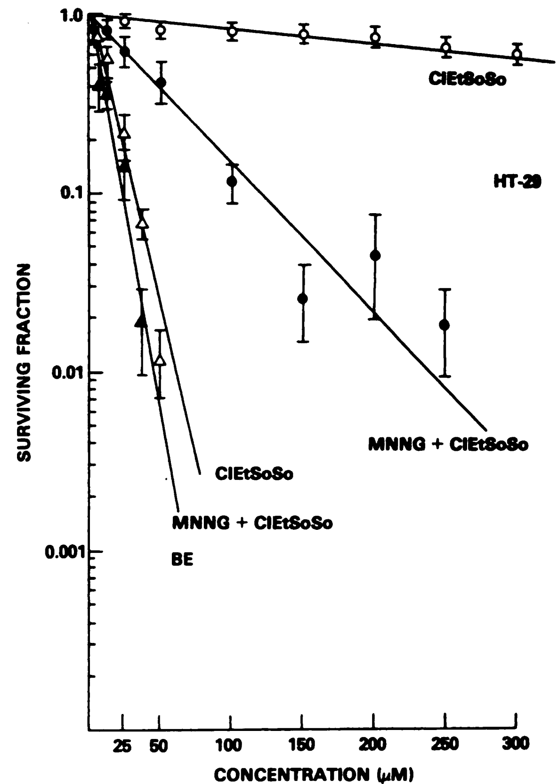

Fig. 1. Inhibition of the colony forming ability of BE (△, ▲) and HT-29 (○, ●) colon carcinoma cells by a 2-h ClEtSoSo exposure with (▲, ●) or without (△, ○) a 1-h MNNG pretreatment. *Points,* means of three or more independent replicate flasks in the same experiment; *bars,* SD.

cells after pretreatment with MNNG was associated with the formation of DNA interstrand cross-links by both ClEtNus (9) and mitozolomide (10).

One possible explanation for the differential cytotoxicity observed between the BE and HT-29 carcinomas would be that drug uptake is different in the two cell lines. It has been shown previously that the quantity of DNA-protein cross-linking in two comparable cell lines is equivalent at equimolar concentrations of ClEtSoSo (3). Thus drug uptake and intracellular reactivity would be similar in both cell lines. In addition, pretreatment of the HT-29 cells with MNNG does not alter the quantity of DNA-protein cross-links formed after exposure to ClEtSoSo (Fig. 3). This is in good agreement with data previously obtained for the ClEtNus and mitozolomide (9, 10).

ClEtSoSo has been shown previously to induce a complex pattern of DNA single strand breaks (3). In addition, pretreatment with 2 μM MNNG has been shown to cause a quantity of strand breaks similar to that produced by 150 rads of γ-rays (9). Fig. 4 shows the production of DNA single strand breaks in the HT-29 cell line by ClEtSoSo either alone or following pretreatment with 2 μM MNNG. The possibility cannot be discounted that the significant number of strand breaks produced may mask the detection of any DNA interstrand cross-links by the alkaline elution assay even though the MNNG induced breaks are accommodated for by irradiation with only 150 rads in the cross-link assay. Both ClEtNus and mitozolomide produce DNA single strand breaks but in these cases interstrand cross-links were detected following MNNG pretreatment using a similar experimental protocol (9, 10).

Pretreatment with the methylating agent STZ has also been shown to induce increased cell killing and the production of interstrand cross-links in a Mer⁺ cell line,[3,4] This agent has several potential advantages over MNNG as a pretreatment

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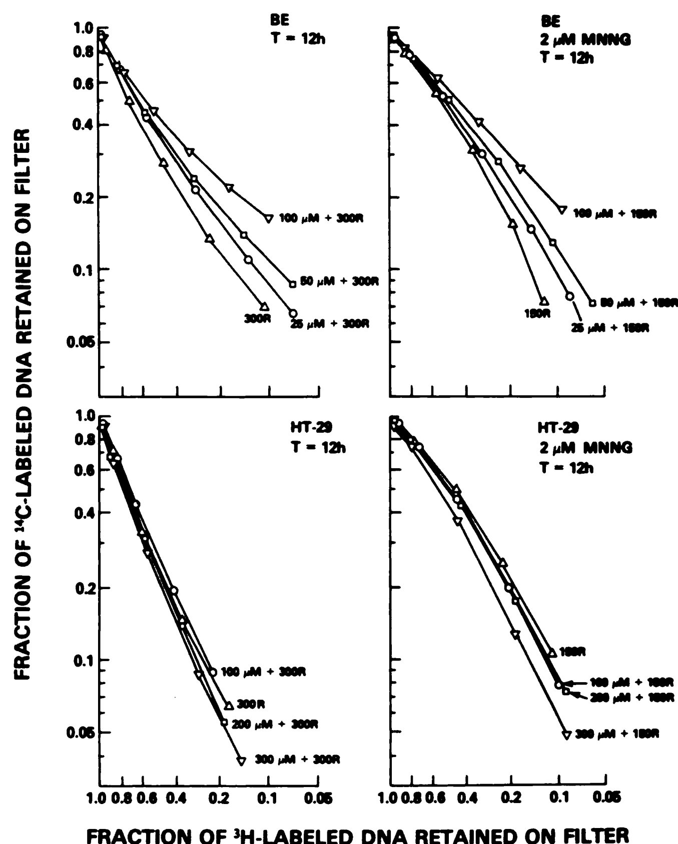

Fig. 2. Alkaline elution assays to test for the formation of DNA interstrand cross-links 12 h after a 2-h ClEtSoSo treatment of BE and HT-29 colon carcinoma cells. The effect of a 1 hour pretreatment of BE and HT-29 with MNNG followed by a 2-h ClEtSoSo exposure is shown. In all experiments the alkaline elution assays are performed 12 h after a 2-h ClEtSoSo exposure. These profiles are taken from one experiment and are representative of three independent experiments.

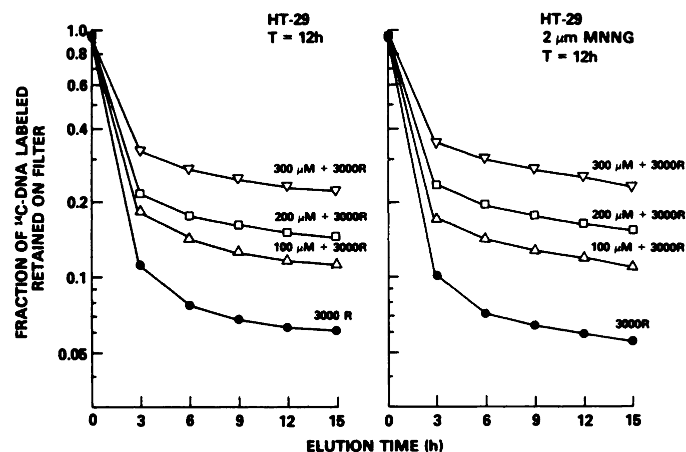

Fig. 3. Alkaline elution assays to test for the formation of DNA-protein cross-links in HT-29 cells after ClEtSoSo or with prior MNNG pretreatment. In these experiments the ClEtSoSo exposure was for 2 h with a 1-h methylating agent pretreatment when appropriate. Alkaline elution assays were performed 12 h after drug treatment. These profiles are taken from one experiment and are representative of three independent experiments.

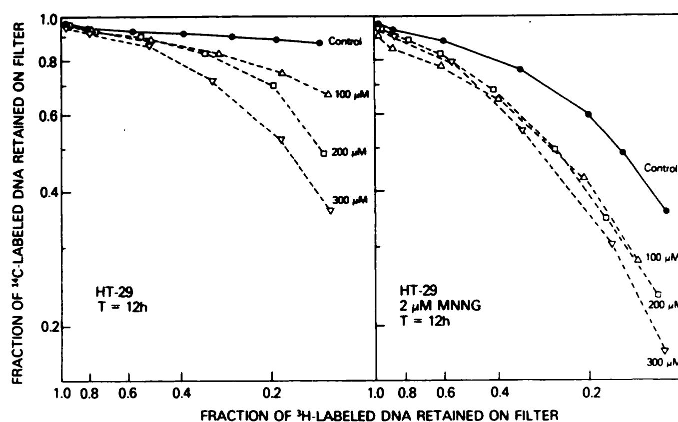

Fig. 4. Alkaline elution assays to detect DNA single strand breaks in HT-29 cells by ClEtSoSo (*left*) or with prior MNNG pretreatment (*right*). In these experiments the ClEtSoSo exposure was for 2 h with a 1-h methylating agent pretreatment when appropriate. Alkaline elution assays were performed 12 h after drug treatment. These profiles are taken from one experiment and are representative of three individual experiments.

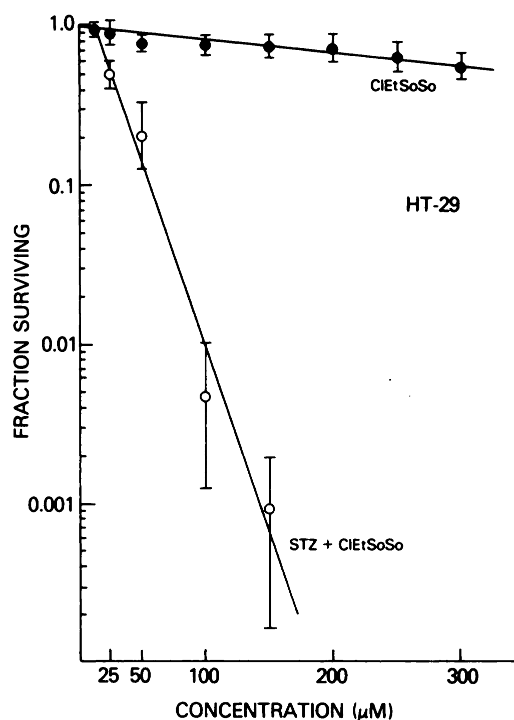

Fig. 5. Inhibition of the colony forming ability of HT-29 cells by ClEtSoSo with (O) or without (●) STZ pretreatment. *Points*, means three or more independent replicate flasks in the same experiments; *bars*, SD.

agent, *e.g.*, its clinical usefulness and its decreased cytotoxicity and carcinogenicity. Fig. 5 shows that pretreatment with 2.5 mM STZ, a dose previously shown to be nontoxic,[4] increases the cell killing of the Mer$^+$ HT-29 cell line to ClEtSoSo in a manner similar to pretreatment with 2 μM MNNG. The combination of STZ and ClEtSoSo also produced a high level of DNA single strand breakage in the HT-29 cell line (Fig. 6, *left*). In addition, pretreatment of the HT-29 cells with STZ induced DNA interstrand cross-linking at doses of ClEtSoSo that failed to produce such cross-links following MNNG pretreatment. It is interesting to note that the nontoxic level of STZ used to pretreat the cells produced a greater number of DNA single strand breaks than the pretreatment dose of MNNG. Since the cross-links are clearly detected in the cross-link assay and even produce an increased retention of the DNA in the strand break assay in Fig. 6, it is unlikely that cross-links were present but undetected in the MNNG pretreatment experiments.

A fast elution method has been developed to overcome some of the problems of strand break interference in DNA interstrand cross-link assays. Because many DNA strand breaks increase with time, resulting in convex elution curves, a fast elution assay enables the detection of interstrand cross-links before many of the strand breaks have formed. In this assay the DNA is eluted through the filter at 3 times the rate of the normal

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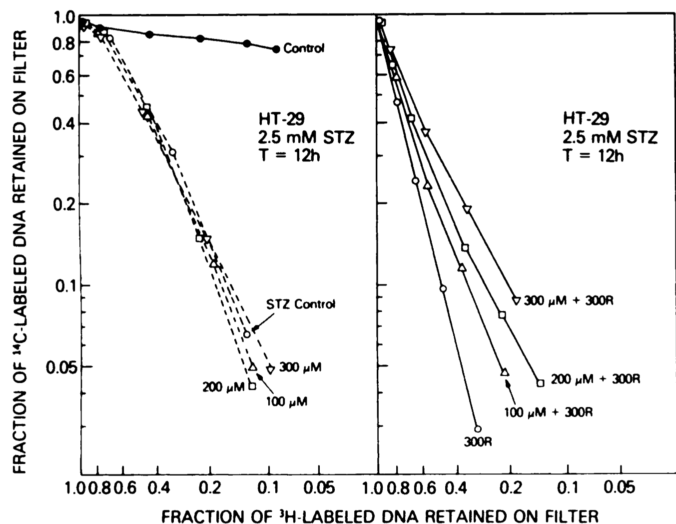

Fig. 6. Alkaline elution assays to detect DNA single strand breaks (*left*) and DNA interstrand cross-links (*right*) in HT-29 cells by ClEtSoSo with STZ pretreatment. In these experiments the ClEtSoSo exposure was for 2 h with a 1-h methylating agent pretreatment when appropriate. Alkaline elution assays were performed 12 h after drug treatment. These profiles are taken from one experiment and are representative of three individual experiments.

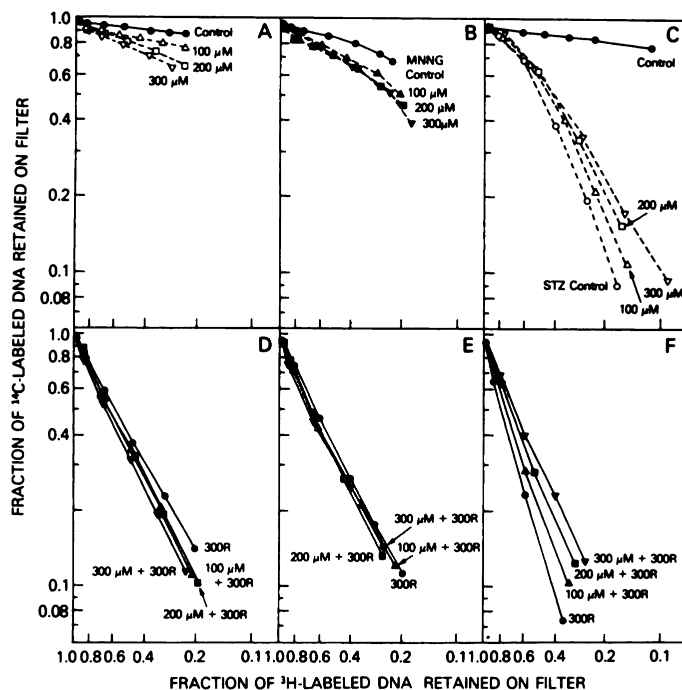

Fig. 7. "Fast" alkaline elution assay to detect DNA single strand breaks (*A–C*) and DNA interstrand cross-links (*D–F*) in HT-29 cells by ClEtSoSo alone (*A, D*), with MNNG pretreatment (*B, E*), or with STZ pretreatment (*C, F*). In these experiments the ClEtSoSo exposure was for 2 h with a 1-h methylating agent pretreatment when appropriate. Alkaline elution assays were performed for 12 h after drug treatment. These profiles are taken from one experiment and are representative of three individual experiments.

assay. Fig. 7 shows the fast elution assay of ClEtSoSo induced strand breaks and interstrand cross-links either alone or following MNNG or STZ pretreatment. It can be seen that using this procedure the number of strand breaks produced by both MNNG and STZ is significantly reduced (Fig. 7, *top*). Nevertheless, this reduction in strand breaks does not reveal any interstrand cross-links in the MNNG pretreated HT-29 cells, although the STZ pretreated cells again show a significant, dose dependent increase in DNA interstrand cross-links.

DISCUSSION

Pretreatment of human tumor cells, proficient in the repair of guanine O^6 alkylations (Mer⁺), with methylating agents such as MNNG or STZ enhances the cell killing effects of chloroethylating agents. This increase in cytotoxicity has been suggested to be due to the saturation of the guanine O^6-alkyltransferase activity by the methylating agent pretreatment and this prevents the removal of guanine O^6-chloroethyl monoadducts produced by the chloroethylating agent. These guanine O^6-chloroethyl monoadducts or the resultant DNA interstrand cross-link are the two most likely candidates responsible for the increased cytotoxicity. In this study we investigated the effect of either MNNG or STZ pretreatment on the cytotoxicity and DNA interstrand cross-linking ability of ClEtSoSo, a novel antitumor agent which may soon enter phase I clinical trial in humans.

Consistent with previous observations with ClEtNus (9)[3], both MNNG and STZ pretreatment of HT-29 colon carcinoma cells increased the cytotoxicity of ClEtSoSo. In contrast to results with ClEtNus (9), no DNA interstrand cross-links were observed with ClEtSoSo after MNNG pretreatment; however, STZ pretreatment allowed the formation of DNA interstrand cross-links by ClEtSoSo in HT-29 cells. To help explain these complicated results three main points might clarify their understanding: (*a*) the mechanism of action of ClEtSoSo may differ from that of other chloroethylating agents such as ClEtNus and mitozolomide; (*b*) the mechanism by which the methylating agent pretreatment operates may differ; (*c*) the formation of DNA strand breaks in the alkaline elution assay may interfere with the detection of DNA interstrand cross-links.

Initial experiments with ClEtSoSo suggested that it have the same basic mechanism as ClEtNus or mitozolomide (3, 9, 10), in that DNA interstrand cross-links were produced in Mer⁻ cells but not Mer⁺ cells and this correlated with the increased toxicity of each agent to Mer⁻ cells. High pressure liquid chromatography analysis of drug treated calf thymus DNA indicated that ClEtNus were capable of both hydroxyethylation and chloroethylation, whereas ClEtSoSo only performed chloroethylation reactions (4). Hence ClEtSoSo was a more selective alkylating agent than ClEtNus. Furthermore, DNA sequence analysis showed that ClEtSoSo alkylated the N^7 position of guanine without any degree of specificity yet ClEtNus reacted preferentially with the middle guanine in runs of three or more (5). These data highlight certain differences which exist betwen ClEtSoSo and ClEtNus and may help to account for those obtained in this study.

The mechanism by which methylating agents inactivate the repair of an O^6-alkylguanine lesion is not yet fully understood. Since, however, it has been reported that the cell contains a finite amount of the repair protein (16) it seems that at least two pathways are possible: (*a*) the methylating agent may be able to react with the repair protein itself, thus inactivating it directly; or *b*) the methylating agent acts by alkylating DNA, and then the protein which reacts with the alkylated guanine in a stoichiometric manner is simply depleted. In addition, a combination of these two mechanisms may also occur. Both MNNG and STZ are known to methylate DNA efficiently and to exhibit comparable levels of methylation at the guanine O^6-position (17, 18). It may be, however, that differences between MNNG and STZ exist in the inactivation of the repair protein which could explain the different results obtained with MNNG and STZ here.

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The technique of alkaline elution suffers from the problem that DNA strand breaks may interfere with the detection of DNA interstrand cross-links and *vice versa* (15). Hence the fact that no interstrand cross-links are detected after MNNG pretreatment with ClEtSoSo in the Mer$^+$ cell may reflect such a situation. STZ, however, appears to produce more DNA strand breaks than MNNG and in this case DNA interstrand cross-links are detected. Moreover, the development of a fast elution methodology capable of minimizing DNA strand break interference produced results identical to those obtained with the classical elution assay. The possibility that the ratio of strand breaks to DNA interstrand cross-links allowed the detection of ClEtSoSo induced cross-links after STZ pretreatment but not MNNG pretreatment appears unlikely but cannot be excluded.

The results obtained in this study identify certain problems that can be encountered in the detection of DNA damage after the exposure of cells to two or more agents. Additionally, they raise the possibility that the cytotoxicity of chloroethylating agents may be due to the persistence of the O^6-chloroethyl monoadduct rather than DNA interstrand cross-link formation. In conclusion, the biological consequences of the methylating agent-chloroethylating agent regimen, irrespective of the mechanisms involved, results in an increased cell kill to tumor cells previously resistant to ClEtSoSo.

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