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O^6-Methylguanine-DNA methyltransferase activity and induction of novel immunogenicity in murine tumor cells treated with methylating agents*

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Summary. To investigate the mechanism of the generation of immunogenic tumor variants by mutagenic drugs, murine leukemia cells exhibiting different sensitivity to killing by the alkylator 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and different ability to repair O^6-methylguanine in their DNA were treated in vitro with a series of methylating agents, including triazene derivatives, temozolomide, and streptozotocin. At the population level, we found that BCNU-resistant cells (L1210/BCNU) that appeared to be cross-resistant to killing by a dimethyltriazene and expressed high levels of O^6-methylguanine-DNA methyltransferase activity (mer$^+$ phenotype) failed to generate highly immunogenic variant sublines on repeated exposure to the methylating agents. In contrast, all cells (L1210) that were susceptible to DNA alkylation damage and deficient in O^6-methylguanine repair (mer$^-$) developed immunogenic variant sublines. A noticeable exception was represented by streptozotocin treatment, which was equally effective in mer$^+$ and mer$^-$ cells. At the clonal level, a single exposure to streptozotocin or a triazene derivative resulted in a high incidence (33% and 50%, respectively) of immunogenic cell generation in mer$^-$ cells only. In mer$^+$ cells, streptozotocin treatment led to a 33% incidence of immunogenic clones only when the cells were concurrently exposed to O^6-methylguanine as a free base. The activity of O^6-methylguanine-DNA methyltransferase in mer$^+$ cells was greatly reduced by treatment with O^6-methylguanine or streptozotocin, and the combination of the two drugs led to enzyme levels similar to those observed in mer$^-$ cells. Taken together, these data suggest that the mechanism of O^6-alkylation may be operative in the induction of novel tumor-cell antigenicity by methylating agents.

Introduction

Highly immunogenic ("xenogenized" or "tum$^-$") cell variants are generated by the treatment of murine tumors with potent mutagens such as the triazene [3] and nitrosoguanidine [4] derivatives via mechanisms resulting in the appearance of novel antigenic specificities that are not detectable on the original tumor [25]. Evidence suggests that the immunogenic molecules newly expressed by the drug-treated tumor cells represent the mutagenized variants [1, 8, 15] of cellular proteins recognized by the host immune system; in the case of the triazene-xenogenized L5178Y lymphoma line, these proteins have been identified as structurally abnormal glycoproteins that are coded for by endogenous retroviral sequences [17]. In general, it is assumed that genomic point mutations leading to single or multiple amino-acid changes in constitutive cellular proteins may generate new immunogenic epitopes on xenogenized variant cells [1, 5, 12, 17].

Although the mode of action of triazene derivatives has not been clarified in detail, one major hypothesis [13, 14, 21, 26] is that these agents either represent or lead to the formation of methylating species that are capable of producing cytoreductive [13, 14, 21, 26], mutagenic [20], and xenogenizing [23] effects. Studies on the interaction of alkylating species with DNA have revealed at least 12 distinct sites in DNA that can act as major targets for alkylation [24], some of which appear to be crucially involved in the mutagenic and carcinogenic effects of the drugs [10, 24]. In particular, substantial evidence has been obtained that methylation of the O^6-position of guanine plays a major role in the mutagenic activity of methylating agents. In fact, the enzyme that removes this DNA adduct,

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MT, methyltransferase; O6-mG, O^6-methylguanine; TZM, temozolomide; DM-Cl, 1-(*p*-chlorophenyl)-3,3-dimethyl-triazene; DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; STZ, streptozotocin; MST, median survival time

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a methyltransferase (MT), was found to counteract the mutagenic properties of the agents [10, 18], and a positive correlation could be established between the MT content and the resistance of different tissues to mutagenesis by those drugs [9]. Indeed, alkylation of the O^6-position of guanine makes this base prone to mispairing with a thymine, and after a subsequent cell division and DNA replication, a GC:AT transition may occur [10].

The main objectives of the present study were to ascertain whether alkylation of the O^6-position of guanine and the resulting transition play a role in triazene xenogenization and whether agents other than triazenes that are known to produce this type of DNA damage are also endowed with xenogenizing properties.

The level of MT is cell-type- and species-dependent, and in tissues rich in MT activity, the O^6-methyl adduct can be removed before DNA replication, thus preventing mutation. Among the established human and murine cell lines, several are extremely sensitive to alkylating agents and are deficient in activity for the removal of O^6-methylguanine (O^6-mG) from DNA. This character has been called mer$^-$ [7] or mex$^-$ [27]. Mer$^+$ or mex$^+$ cell lines have been shown to contain high levels of O^6-mG-DNA MT activity. Using tumors exhibiting different sensitivity to killing by an alkylator and different ability to repair O^6-mG in their DNA, in the present study we investigated the involvement of this adduct in tumor-cell xenogenization by triazene derivatives and other methylating agents. The study was based on the premise that if O^6-mG were to play a role, mer$^-$ and mer$^+$ tumor-cell lines would respond differently to drug-induced xenogenization.

Materials and methods

Animals. Hybrid (BAB/c Cr×DBA/2) F1 (CD2F1, *H-2*^{d}/*H-2*^{d}) mice were obtained from Charles River Breeding Laboratories (Calco, Milan, Italy). Mice of both sexes, ranging in age from 2 to 4 months, were used.

Tumors. L1210 and L1210/*N,N*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) mouse leukemia cells (Frederick Cancer Research Facility, Frederick, Md.) were maintained in vitro as tissue cultures in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, and antibiotics.

Drugs. Temozolomide (TZM) was kindly provided by Dr. C. G. Newton (May & Baker Ltd., Dagenham, Essex; RM107XS) and was dissolved in 0.5% dimethyl sulfoxide immediately before its use. 1-(*p*-Chlorophenyl)-3,3-dimethyl-triazene (DM-Cl) was obtained as described elsewhere [23]. 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) and streptozotocin (STZ) were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, Md.). O^6-mG was synthesized by the method of Balsinger and Montgomery [2] using 6-chloroguanine from Sigma Chemical Co. (Saint Louis, Mo.).

Drug treatment of tumor cells and cloning. L1210 and L1210/BCNU tumor cells (2×10^6 cells/ml) were exposed in vitro to 0.4 mM TZM [6], 4 mM DTIC [23], 3 mM DM-Cl [23], or 4 mM STZ for 1 h at 37° C in an atmosphere containing 5% CO_2. These drug concentrations were selected as being roughly equitoxic to L1210 cells in vitro. Cell treatments with DTIC or DM-Cl were carried out in the presence of a mouse liver preparation for metabolic activation of drug as previously described [23]. Treatment of cells with O^6-mG was performed by incubating tumor cells with 0.4 mM O^6-mG for 24 h [28], with appropriate concentrations of methylating agents being added during the final hour of incubation. After treatment with the different drugs, tumor cells were either used as bulk populations in the different in vitro and in vivo assays or allowed to regrow for 1 week prior to cloning by limiting dilution in flat-bottomed 96-well microtiter plates [12]. In the experiments on repeated mutagenesis, in-vivo-passaged (in irradiated recipients) tumor cells were treated with the drugs immediately prior to each serial transplantation [23].

Assessment of immunogenicity. Tumor-cell bulk populations or individual clones of untreated or drug-treated L1210 or L1210/BCNU cells were injected (10^5) i.p. into intact or 400-rad-irradiated mice (six to eight animals per group) that were followed for mortality parameters for at least 60 days. Results were expressed as the median survival time (MST) of the tumor-challenged mice or as the difference in MST between the intact and the irradiated tumor recipients (ΔMST) [12].

MT assay. The MT assay was performed as reported elsewhere [22] using crude cellular extracts and [^{3}H]-methyl-labelled DNA, whereby the amount of enzyme-associated S-labeled cysteine formed as a result of MT activity was measured. Briefly, extracts of drug-treated cells were prepared by sonication, and methylated standard DNA containing about 500 fmol O^6-mG was incubated with variable amounts of extracts in 0.8 ml reaction mixture containing 1.7 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 mM phenylmethylsulfonylfluoride at pH 8. Samples were incubated either with or without cell extracts (control) at 37° C for 1 h, and the mixture was then treated with 2.5 vol. cold 6% trichloroacetic acid. The resulting precipitate, which also contained radioactive DNA, was hydrolyzed by heating at 80° C for 30 min and collected on glass-fiber GP/C filters following the addition of a bovine serum albumin carrier. The filters were washed and dried and treated with a tissue solubilizer (Soluene, Packard), and the radioactivity was counted using a scintillation cocktail with very high chemiluminescence decay (Hionic-Fluor, Packard).

Statistical analysis. In the in vivo tumor-challenge experiments, each experimental group consisted of six to eight animals and mortality data were analyzed using the Mann-Whitney *U*-test. Student's *t*-test was used for analysis of the results of the in vitro determinations of MT activity.

Results

MT activity in L1210 and L1210/BCNU lines

L1210/BCNU is a tumor subline of mouse leukemia L1210 that is resistant to both chloroethylnitrosoureas and methyltriazenes [6]. Following preliminary in vitro experiments confirming the differential sensitivity of L1210 and L1210/BCNU to DM-Cl killing (data not shown), we measured MT activity in the two tumor-cell lines. Enzyme activity in the mer$^+$ cells was also measured in the presence of O^6-mG, a well-know inhibitor of O^6-mG-DNA MT [28]. We found that MT activity was significantly higher in L1210/BCNU than in parent cell extracts and that O^6-mG treatment caused a significant reduction in enzyme activity in BCNU-resistant cells (Table 1). Therefore, besides substantiating previous data on the differential MT content of parent and BCNU-resistant cells, these findings indicate that the addition of O^6-mG as a free base may inhibit O^6-mG-DNA MT activity in L1210/BCNU cells.

Table 1. O^6-mG-DNA MT activity[a] in L1210 and L1210/BCNU cells

Tumor	Pretreatment with O^6-mG	
	–	+
L1210	378 ± 960[b]	ND
L1210/BCNU	4,231 ± 1,496*	1,943 ± 925**

[a] MT activity was expressed in fmol/mg DNA
[b] Mean ± SD ($n = 14$)
* $P < 0.01$/($n = 5$, L1210/BCNU vs L1210 cells)
** $P < 0.05$/($n = 4$, O^6-mG-treated vs untreated L1210/BCNU cells)
ND, Not done

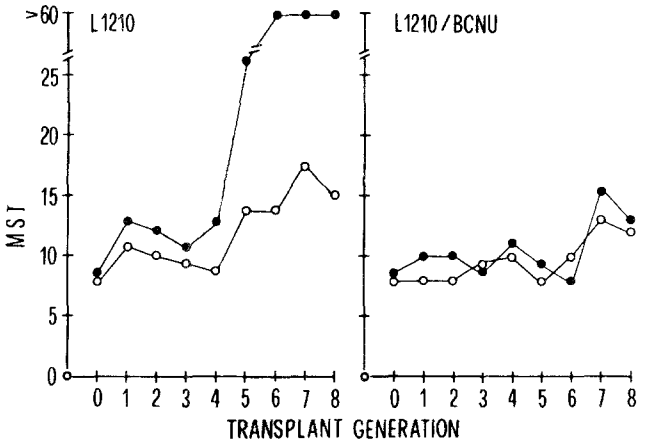

Fig. 1. Xenogenization of parent and BCNU-resistant cells by DTIC. In vivo-passaged (in irradiated hosts) cells of either phenotype were treated with the drug in vitro prior to transplantation in vivo and were injected at each transplant generation into intact (—●—) or immunodepressed (—○—) mice (6–8/group) for detection of immunogenicity. Mortality data were recorded for at least 60 days and were expressed as the MST of the tumor-challenged mice in days

Xenogenization of L1210 and L1210/BCNU tumor cells by DTIC

Most studies on the xenogenization of murine tumors have been carried out using the dimethyltriazene DTIC [25]. For this reason, we decided to investigate the susceptibility of L1210 and L1210/BCNU cells to xenogenization in vitro by this drug. According to a previously described procedure based on a repeated-mutagenesis protocol [23], the cells were treated in vitro with the drug over the course of several transplant generations, with each round of DTIC exposure (prior to in vivo grafting) involving the conditions described in Materials and methods. At each generation, the drug-treated cells were injected into intact or immunodepressed hosts for detection of immunogenicity. Under these conditions, the difference in MST between intact and immunodepressed mice is a measure of tumor-cell immunogenicity [12]. Figure 1 shows that the treatment of L1210 cells with DTIC resulted in a progressive increase in tumor immunogenicity such that at transplant generation 6, the drug-treated cells were no longer tumorigenic in intact recipients. In contrast, L1210/BCNU cells failed to acquire immunogenic properties following treatment with DTIC for up to eight transplant generations. This suggested that the mer+ character might negatively influence the induction of immunogenicity by DTIC.

Table 2. Xenogenization of L1210 (mer⁻) and L1210/BCNU (mer+) cells by different drugs

Cell type	Drug[a]	ΔMST[b] (days) at					
		TG0	TG2	TG4	TG6	TG8	TG10
mer⁻	DTIC	0	2	4*	>45*	>45*	>45*
mer⁻	DM-Cl	1	>45*	>45*	>45*	>45*	>45*
mer⁻	TZM	−1	1	3*	2	7*	12*
mer⁻	STZ	1	1	>45*	>45*	>45*	>45*
mer+	DTIC	1	2	1	−2	1	1
mer+	DM-Cl	1	1	1	10*	>45*	>45*
mer+	TZM	2	0	0	1	−2	1
mer+	STZ	1	1	>45*	>45*	>45*	>45*

[a] Details of drug treatment and experimental procedure are reported in Materials and methods (also see legend to Fig. 1)
[b] Difference in median survival of intact and irradiated tumor recipients at each transplant generation (TG). TG0 indicates non-drug-treated tumor cells from which each drug-treated subline was derived. In control sublines treated with medium alone (or medium plus mouse liver preparations) in the absence of any drug, ΔMST never exceeded 2 days
* Differences statistically significant ($P < 0.05–0.01$, intact vs irradiated mice)
Negative values indicate MST (intact) <MST (irradiated mice)

Xenogenization by other methylating agents

Subsequent xenogenization studies based on the same experimental design described for DTIC were conducted using other methylating agents, namely, DM-Cl, TZM (an analogue of DTIC that spontaneously decomposes in vitro to the active metabolite), and STZ. Mer⁻ and mer+ L1210 cells were tested for susceptibility to xenogenization by these drugs in comparison with DTIC, and the results were expressed as ΔMST, i.e., the difference in MST (in days) between tumor-challenged intact and irradiated hosts at each transplant generation. Table 2 shows that all of the drugs could increase the immunogenicity of mer⁻ cells, albeit with different degrees of efficacy. Thus, the xenogenizing potential of DM-Cl and STZ was apparently much greater than that of TZM under the selected treatment conditions. In contrast, on repeated drug exposure, L1210/BCNU cells either failed to acquire strong immunogenicity (DTIC, TZM) or developed it only after many rounds of drug treatment (DM-Cl). A remarkable exception was represented by treatment with STZ, which apparently xenogenized the two types of tumor cell equally well. These data indicated that the BCNU-resistant cells were as a rule less susceptible than the parent cells to xenogenization by methylating agents when bulk tumor-cell populations were repeatedly exposed to these drugs in vitro.

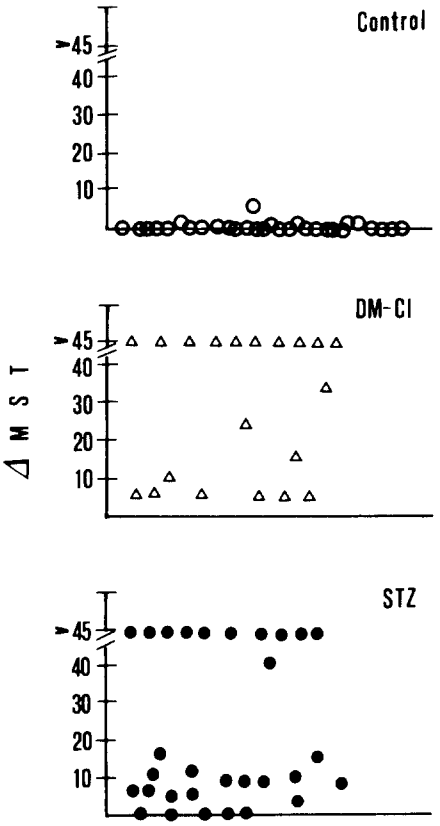

Fig. 2. Xenogenization of parent cells at the clonal level. Control or drug-treated cells were cloned by limiting dilution and the immunogenicity of the resulting clones was determined by injecting 10^5 cells of each clone into 4 intact or immunodepressed recipient mice. Results were expressed as the difference in MST between the intact and the immunodepressed recipients in days

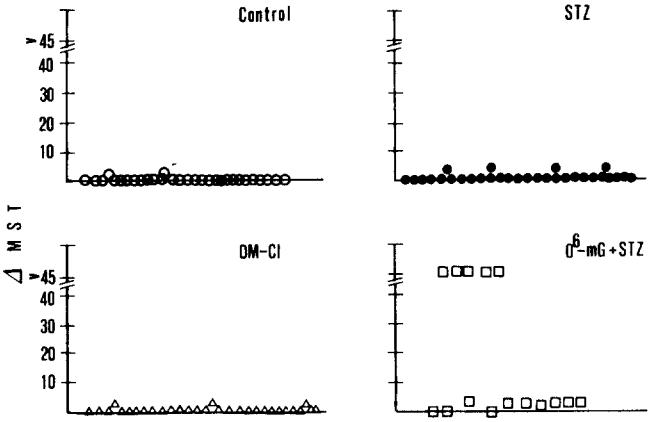

Fig. 3. Xenogenization of BCNU-resistant cells at the clonal level. Control or drug-treated cells were cloned by limiting dilution and the immunogenicity of the resulting clones was determined by injecting 10^5 cells of each clone into 4 intact or immunodepressed recipients. Results were expressed as the difference in MST between the intact and the immunodepressed recipients in days

Table 3. O^6-mG-DNA MT activity in L1210/BCNU cells

Treatments	%MT activity[a]
O^6-mG	32.2 ± 10.1[b]*
STZ	25.2 ± 10.4[c]*
O^6-mG+STZ	10.9 ± 7.9[d]*, **
DM-Cl	104.1 ± 64.8[e]

[a] Mean values for drug-treated cells are expressed as a percentage of the MT activity of control cells. The mean (± SD) MT activity in control cells was 4,385 ± 943 fmol/mg DNA ($n = 6$)
[b] $n = 3$
[c] $n = 8$
[d] $n = 5$
[e] $n = 7$
* $P < 0.01$ vs control cells
** $P < 0.05$ (O^6-mG+STZ vs STZ alone)

Xenogenization of L1210 and L1210/BCNU cells at the clonal level

The susceptibility of mer⁻ and mer⁺ cells to drug-induced xenogenization was also analyzed at the clonal level following a single exposure of tumor cells to the drugs. To this end, bulk cell populations were treated in vitro with DM-Cl or STZ, and this was followed by cell cloning by limiting dilution. Prior to STZ treatment, mer⁺ cells were also treated with O^6-mG. Non-drug-treated tumors were cloned as well. The resulting clones were injected into intact or irradiated recipients whose mortality parameters were recorded for at least 60 days. MST values were calculated; the difference in MST of the intact and the irradiated recipients for each of the clones tested are reported in Figs. 2 (L1210) and 3 (L1210/BCNU). For the mer⁻ L1210 line, it was apparent that 0/30 untreated, 33% (10/30) of the STZ-treated, and 50% (10/20) of the DM-Cl-treated clones were highly immunogenic, with the difference in MST exceeding 45 days. When the examination was extended to mer⁺ cells, it was found that 0/30 untreated and 0/30 DM-Cl-treated clones were immunogenic (Fig. 3). This was also the case for STZ treatment. However, when STZ treatment was preceded by tumor-cell exposure to O^6-mG, the incidence of immunogenic clones rose from 0 (0/30) to 33% (5/15), a value similar to that found for mer⁻ cells treated with STZ alone.

MT activity in L1210/BCNU cells treated with STZ or DM-Cl

We also investigated the way in which STZ treatment either alone or in combination with O^6-mG would affect the expression of MT activity in mer⁺ cells. In fact, it is known that STZ may inhibit the enzyme [16], and this effect in our xenogenization model could be maximized by sequential exposure to O^6-mG and STZ. In the presence or absence of preincubation with O^6-mG for 24 h, L1210/BCNU cells were treated with STZ and then assayed for O^6-mG-DNA MT activity. In addition, we measured enzyme levels following tumor-cell treatment with DM-Cl (Table 3). It was found that under our experimental conditions, STZ or O^6-mG treatment reduced enzyme activity to a similar extent. The combination of the two drugs resulted in levels of MT activity that were comparable with

those found in mer⁻ cells (see Table 1). In addition, Table 3 shows that DM-Cl treatment exerted no inhibitory activity on O⁶-mG-DNA MT levels.

Discussion

Besides possessing cytoreductive properties, triazene and nitrosoguanidine derivatives exhibit potent mutagenic activity, which presumably accounts for most of their ability to generate highly immunogenic variant tumor sublines [1, 3–5, 8, 12, 15, 17, 25]. Such xenogenized variants arise when mouse tumor-cell lines are treated in vitro with the drugs, a procedure that results in a very high incidence (3%–90% of surviving cells) of immunogenic clones and is associated with the detection of new transplantation antigens [5, 12, 25]. It is now believed that any point mutations leading to amino-acid substitutions in cellular proteins or peptides that are accessible to the host immune system may result in changes in the antigenic profile of the drug-treated tumor cells [1, 8, 17].

Alkylation damage to DNA may be both a mechanism of action of methylating agents and a key event in drug-induced mutagenesis. The methylpurine O⁶-mG has long been recognized as being potentially mutagenic because of its miscoding properties [18]. O⁶-mG in DNA is repaired in both bacterial and mammalian cells by O⁶-mG-DNA MT, which removes the methyl group from the methylpurine without excising the guanine base [11, 19]. The "suicide" kinetics of the repair reaction indicates that each cell has a limited capacity to repair O⁶-mG lesions, after which RNA and protein synthesis are required to restore the repair enzyme levels [24]. For this reason, mer⁺ cells can be sensitized to chloroethylnitrosourea-induced killing by procedures known to exhaust the cellular levels of transferase, such as treatment with a nitrosoguanidine derivative [29] or with O⁶-mG as a free base [28].

To clarify the involvement of O⁶-mG in tumor-cell xenogenization by methylating agents, particularly triazene derivatives, we comparatively analyzed drug-induced immunogenic changes in BCNU-sensitive and -resistant murine leukemia cells. On assaying mer⁻ bulk populations, we found that repeated treatment with any of the drugs tested (DTIC, DM-Cl, TZM, or STZ) led to increased tumor-cell immunogenicity according to a pattern and potency that varied from drug to drug. In contrast, mer⁺ cells could be xenogenized only by a high number of DM-Cl treatments or by exposure to STZ. When the examination was carried out at the clonal level, treatment with DM-Cl or STZ resulted in a high incidence of immunogenic clones (50% and 33%, respectively) in mer⁻ cells, whereas no such clones could be generated by exposure of mer⁺ cells to either drug. However, sequential treatment of the latter cells with O⁶-mG and STZ resulted in an incidence of immunogenic clone generation that was similar to that found in mer⁻ cells treated with STZ alone.

The MT transferase protein, which reacts in a stoichiometric manner, is depletable [24]. Moreover, unlike DM-Cl, STZ is a potent inhibitor of the repair mechanisms of O⁶-mG lesions. Two mechanisms that may underlie this effect have been proposed: STZ may directly inactivate the repair protein itself or may simply act by producing high amounts of O⁶-mG, thus saturating out the repair activity [16]. By measuring enzyme levels in the drug-treated tumor, we obtained direct evidence that STZ treatment impairs MT activity in L1210/BCNU cells. This was at variance with the results of similar determinations in cells treated with DM-Cl, which proved that this drug exerts no obvious inhibitory activity on MT levels. Taken together, these data could help to explain the xenogenizing efficacy of repeated STZ treatment of a bulk tumor-cell population that nonetheless expressed high constitutive levels of repair activity.

In addition, these considerations suggested that the high incidence of immunogenic clone generation in mer⁺ cells treated with O⁶-mG and STZ might result from the combined effects of the two drugs on the repair activity. Evidence in this direction came from the finding that the MT activity in mer⁺ cells treated with O⁶-mG plus STZ was similar to that in repair-deficient cells. Therefore, it seems reasonable to hypothesize that the inactivation of MT activity may contribute to the great xenogenizing ability of STZ. In this regard, it is interesting that *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a most potent and widely investigated drug with xenogenizing properties [1, 4, 5, 8], also inhibits the activity of the repair enzyme [29].

In conclusion, the present data suggest an important involvement of O⁶-mG in the xenogenizing activity of methyltriazenes. In addition, for the first time, definite evidence has been provided that the induction of novel immunogenicity in murine tumor cells is not limited to selected alkylating agents such as triazene and nitrosoguanidine derivatives but may rather be a property that is shared by a variety of agents (e.g., STZ) capable of causing alkylation damage to DNA, particularly O⁶-mG lesions. Unlike *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, which displays highly carcinogenic and toxic potential, both methyltriazenes and STZ are used in the clinic because of their appreciable antitumor activity. Undoubtedly, the notion that the antigenic profile of tumor cells may be altered as a result of cytoreductive drug treatment might be of relevance for both a more rational use of these agents and the design of new approaches to cancer immunotherapy.

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