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EXPOSURE OF HELA CELLS TO O^6-ALKYLGUANINES INCREASES SENSITIVITY TO THE CYTOTOXIC EFFECTS OF ALKYLATING AGENTS

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Summary. Exposure of HeLa S3 cells to 0.4mM O^6-methylguanine or O^6-n-butylguanine for 24 h led to a substantial decrease in the activity of O^6-alkylguanine-DNA alkyltransferase. Such pretreatment caused a marked increase in the sensitivity of the cells to the cytotoxic effects of the cross-linking alkylating agent 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea and a smaller increase in the sensitivity to N-methyl-N'-nitro-N-nitrosoguanidine. These results indicate that the repair of DNA by the alkyltransferase plays an important role in the protection of cells from the cytotoxic effects of certain alkylating agents particularly those such as 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea which ultimately lead to the formation of lethal interstrand cross-links.

O^6-Alkylguanine residues in DNA are repaired by the action of a protein termed O^6-alkylguanine-DNA alkyltransferase (AGT)* which catalyzes the transfer of the alkyl group onto a cysteine residue contained within its own protein sequence. Since the alkylcysteine thus formed is not regenerated, the capacity to repair this lesion rapidly is determined by the number of active molecules of the AGT present in the cell (1,2). Once these have been used up, further repair of alkylated DNA requires the de novo synthesis of the AGT protein.

Numerous studies have demonstrated that AGT plays an important role in the prevention of carcinogenesis and mutagenesis in cells exposed to alkylating agents (2-5). In addition, experiments in which cell lines in culture lacking AGT activity (termed mer^-) have been compared to cells having activity

*Abbreviations used: CCNU, 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; C.F.E., colony forming efficiency; AGT, O^6-alkylguanine-DNA alkyltransferase; m^6G, O^6-methylguanine; n-bu^6G, O^6-n-butylguanine.

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(mer^+) have suggested that the protein may protect cells from the cytotoxic effects of both monofunctional and bifunctional alkylating agents (5-12). However, the basic defect in mer^- cells is not well understood, and cells contain a number of enzymes which remove various alkylation products from cellular DNA (1,2). Therefore, it remains possible that other factors which are regulated in parallel with the AGT are responsible for the protective effect. This is clearly the case in E. coli where an adaptive response to alkylation damage turns on not only the AGT but also a glycosylase which removes various N-alkylated purines including 3-methyladenine (1,13,14).

Recently, we and others have found that exposure of cells to O^6-alkylguanines can lead to a loss of AGT activity (15-18). Our results indicate that this loss is brought about by the free base, m^6G, acting as a weak substrate for the AGT. Incubation of the AGT protein from HeLa cells with this base, therefore, leads to its inactivation (17). These observations led to the possibility that exposure of mammalian cells to this base could be used to deplete the AGT activity specifically. The effect of this reduction on the sensitivity of these cells to the cytotoxic effects of alkylating agents could then be tested. This report describes the effects of such treatment in HeLa cells. Very recently, Karran and Williams (18) have described similar experiments with the Raji lymphoma cell line. Their results and conclusions were quite different from ours, and the possible reasons for these discrepancies are discussed.

MATERIALS AND METHODS

Chemicals. CCNU (NSC 79037) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, Bethesda, MD. MNNG and all other biochemicals were purchased from Sigma Chemical Company, St. Louis, MO. The method of Balsiger and Montgomery was used to synthesize n-bu^6G and m^6G (19). The radioactive DNA substrate used for assay of AGT activity was prepared as previously described (20) using N-[^{3}H]-methyl-N-nitrosourea (3.5 Ci/mmol) from Amersham-Searle, Arlington Heights, IL.

Cells. HeLa S3 cells were obtained from the American Type Culture Collection (ATCC CCL2.2) and grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 36mM $NaHCO_3$, 9 units/ml penicillin and 9µg/ml streptomycin. Cells were cultured in a humidified atmosphere (10% CO_2 in air) at 37°C.

Determination of AGT activity. The cells were allowed to grow for 3 days and the medium replaced with fresh medium or fresh medium containing 0.4mM m^6G or n-bu^6G. After 24 h the cells were harvested, extracts prepared and assayed for AGT activity using a partially depurinated [^3H]-methylated DNA substrate as previously described (11,17,20).

Cell growth and survival. This was assayed both by measurement of the cell proliferation and of the ability to form colonies. Cells were seeded at a density of 2 x 10^5 in 25cm^2 flasks and grown for 24 h in the presence or absence of m^6G or n-bu^6G. The cells were then treated with MNNG for 1 h or CCNU for 2 h at the doses indicated in the figure legends. For the cell proliferation studies the cells were then allowed to grow for 2 or 4 days with a change to fresh medium 24 h after treatment with the alkylating agents. (In the experiment shown in Figure 1, Panel D, the fresh medium also contained 0.4mM m^6G but in other experiments the m^6G or n-bu^6G was not renewed at this time). Cell number was determined by counting on an electronic Coulter Counter. For the measurements of colony forming efficiency the cells were replated after drug treatment at densities between 15 and 4500 cells in 60 mm dishes. The colonies formed at 9-11 days later were washed with 0.9% saline, stained with 0.5% crystal violet in ethanol and counted. The plating efficiency for the untreated cells varied between 37 and 69%. It was not affected by the presence of the O^6-alkylguanines in the absence of alkylating agents.

RESULTS

When HeLa S3 cells were cultured for 24 h in the presence of 0.4mM m^6G or n-bu^6G, there was a substantial loss in the AGT activity which could be detected in extracts prepared from these cells (Table 1). In agreement with our previous reports (16,17) about 80% of the activity was lost under these conditions. The cells were, therefore, treated in this way and the effects of MNNG and CCNU on their growth investigated (Figure 1). The results shown in Figure 1 show the cell number measured 4 days after the treatment but similar results were obtained after 2 days of growth. The treatment with m^6G sensitized the cells to the toxic effects of MNNG (Panel A) and CCNU (Panels B and

Table 1. Effect of O^6-Alkylguanines on AGT in HeLa cells

Base added to culture medium	O^6-alkylguanine-DNA alkyltransferase activity (units/mg protein)
None	0.75 (100%)
Guanine	0.78 (104%)
O^6-Methylguanine	0.19 (25%)
O^6-n-Butylguanine	0.15 (20%)

The cells were exposed to 0.4mM concentrations of the base shown for 24 h prior to harvest and the assay of alkyltransferase activity. One unit of alkyltransferase represents the capacity to remove 1 pmol of O^6-methylguanine from an alkylated DNA substrate.

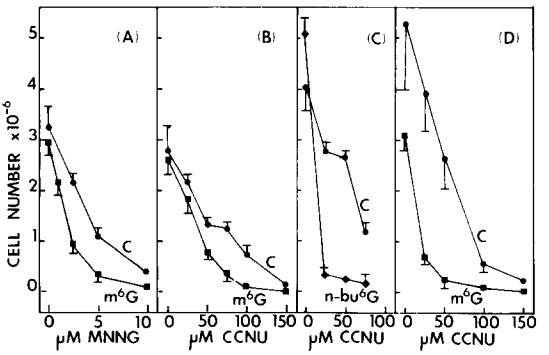

Figure 1. Effect of O^6-alkylguanines on proliferation of HeLa cells treated with CCNU or MNNG. Cells were exposed to 0.4mM m^6G (Panels A, B, D) or 0.4mM n-bu^6G (Panel C) for 24 h and then treated with CCNU for 2 h (Panels B-D) or MNNG for 1 h (Panel A). The cell number 4 days after treatment is shown. In the experiments shown in Panels A-C, the O^6-alkylguanine was removed after 24 h; in the experiment shown in Panel D it was left in for the entire 4 day period. Results for control cells in all panels are indicated by C(● —— ●), results for cells treated with n-bu^6G by ◆ —— ◆ and results for the cells treated with m^6G by ■ —— ■.

D). O^6-n-Butylguanine was also active and may have been somewhat more potent in sensitizing the cells to CCNU (Panel C). As shown in Panels A-C the exposure to the O^6-alkylguanine alone for a total period of 48 h had no significant effect on the cell growth. In the experiment shown in Panel D the cells were exposed to m^6G not only for the 24 h period prior to treatment but also for the subsequent 4 days. This increased the sensitization to CCNU slightly but also affected the growth of the cells not treated with the alkylating agent.

More definitive indication of the cytotoxic effects of the alkylating agents can be obtained by measurements of the cell colony forming ability. These results are shown in Figure 2. It was found that exposure to 0.4mM m^6G greatly increased the effect of CCNU in reducing the colony forming efficiency of the HeLa cells (Panel A). There was also a less marked but significant increase in the reduction of colony forming efficiency by MNNG (Panel B).

DISCUSSION

Although the DNA repair alkyltransferase protein isolated from *E. coli* acts not only on O^6-alkylguanine but also on O^4-alkylthymine and on alkylphosphate triesters (13,21,22), the mammalian AGT is specific for adducts at

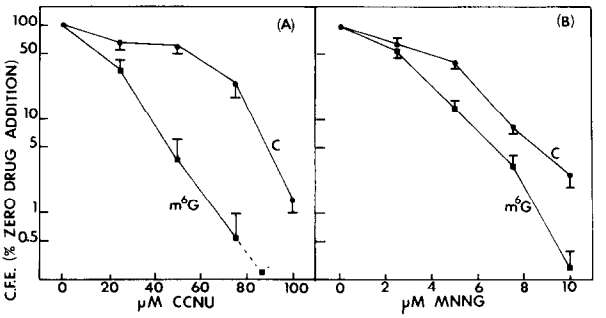

Figure 2. Effect of O^6-alkylguanines on cloning efficiency of HeLa cells. The cells were treated as in the legend to Figure 1 and the colony forming efficiency (C.F.E.) determined and expressed as the percentage of the C.F.E. with no addition of the alkylating agent. The treatment with 0.4mM O^6-methylguanine for 24 h did not affect the C.F.E. of the cells which were not exposed to the alkylating agents. Panel A shows results for CCNU treatment and Panel B for MNNG treatment. Results for control cells are indicated by C (● —— ●) and results for the cells treated with O^6-methylguanine (m^6G) by ■ —— ■. The asterick indicates a C.F.E. of less than 0.1%. The error bars represent 1 S.D. of at least 4 replicate plates.

the O^6 position of guanine (11,24). Therefore, our results showing that the cytotoxicity of alkylating agents are increased in HeLa cells when the AGT capacity is depleted support the concept that O^6-guanine adducts or their derivatives are lesions which have the potential to cause cytotoxicity.

This result is contradictory to the recent report by Karran and Williams (18). They reduced AGT activity in Raji cells by exposure to m^6G but found no effect on the survival upon treatment of these cells with alkylating agents in a single experiment following cell number during growth for 4 days. One possible explanation for this discrepancy may relate to the speed with which the AGT protein is renewed in the different cells investigated. Karran and Williams (18) report that when the m^6G is removed the AGT activity recovers in Raji cells within 4 h. In our experiments with the HeLa cells, full recovery of activity required 48 h (17). This recovery period probably represents the time needed for resynthesis of the AGT protein, and these results suggest that synthesis occurs much more rapidly in the Raji cells. The newly synthesized AGT would be expected to react preferentially with lesions in DNA rather than with the free m^6G since the free base is many orders of magnitude poorer as a substrate than O^6-alkylguanine in DNA (17). Therefore, it is possible that

the resynthesis of the AGT is able to remove most of the lesions in the Raji cells before irreversible damage occurs, whereas in HeLa cells the synthesis rate is not adequate for such removal.

Resynthesis of the AGT may also explain why the effect of exposure to m^6G on the cytotoxicity of CCNU is greater than with MNNG when measured by the C.F.E. assay (Figure 2). It is thought that the toxic lesion produced by agents such as CCNU involves a DNA interstrand cross-link (8,9,24,25). The initial step in the formation of this cross-link is an attack on the O^6-position of guanine forming an adduct at this site which then undergoes a further reaction and rearrangement yielding the potentially lethal cross-link. If the active AGT is present and removes the initial adduct from the O^6-position of guanine prior to the cross-link formation the toxicity is prevented (25-28). Once the cross-link is formed it no longer involves the O^6 position and the AGT cannot repair it. Thus, resynthesis of the AGT may only have an effect if it occurs in the period immediately following exposure to CCNU prior to cross-link formation. In contrast, it is probable that the m^6G formed by MNNG exerts toxic effects by virture of its own ability to interfere with normal DNA synthesis or transcription. Substantial repair of the m^6G may occur by newly synthesized AGT during the time period in which the cellular proliferation occurs prior to the development of discrete clones.

It should be emphasized that many different targets resulting from the attack of alkylating agents on cells can exert lethal effects and that our results merely indicate that the formation of O^6-alkylguanine adducts is one of these. There is clear evidence from experiments with mutant or variant cells possessing similar AGT activities but very different sensitivities to killing by alkylating agents that this is the case (5,10,18). Nevertheless, our results indicate that depletion of the AGT activity can profoundly alter the survival of certain tumor cells exposed to CCNU (Figure 2) and in this way can be used to augment its chemotherapeutic potential.

Our results are consistent with experiments in which Erickson and his colleagues (26-28) have shown that pretreatment of mer^+ tumor cells with MNNG

or N-methyl-N-nitrosourea enhances the toxicity of CCNU presumably by saturating the cellular AGT activity. The use of m^6G or $n\text{-}bu^6G$ to reduce the cellular AGT level rather than a monofunctional alkylating agent might be beneficial since the O^6-alkylguanines have little intrinsic toxicity of their own, whereas the N-nitroso- compounds are highly toxic and are potent mutagens and carcinogens.

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