Adaptive response in mammalian cells: Crossreactivity of different pretreatments on cytotoxicity as contrasted to mutagenicity

(DNA repair/nitrosoureas/alkylating agents/O6-methylguanine/rat hepatoma cells)

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ABSTRACT Pretreatment of H4 (rat hepatoma) cells for 48 hr with low nontoxic doses of alkylating agents [methyl methanesulfonate (MMS), N-methyl-N' nitro-N-nitrosoguanidine (MNNG), and N-methyl-N-nitrosourea (MNU)], an adaptive DNA repair pathway is induced. This adaptive response is demonstrated by the induction of high levels of O6-methylguanine (O6MeGua) in MMS-pretreated cells as compared to control cultures, whereas the disappearance of this lesion is not modified in MMS-pretreated cells. As MMS produces less methylation at the O6 position of guanine and more methylation at the N7 position in comparison to MNNG, these results suggest that (i) N7-methylguanine is not implicated in the adaptive response and (ii) adaptation to mutagenesis can be correlated with the amount of O6-methylguanine induced during the pretreatment. The effect of pretreatment on other O-alkylated derivatives is not known.

When Escherichia coli cells are continuously exposed to nontoxic levels of a simple alkylating agent, such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or N-methyl-N-nitrosourea (MNU), an adaptive DNA repair pathway is induced. This adaptive response is also observed in Micrococcus luteus (4).

In Escherichia coli, different processes are implicated in the adaptive response. Adaptation to killing by alkylating agents is attributed to the induction of high levels of a DNA glycosylase, coded by the alk gene (5), which excises 3-methyladenine, 3-methylguanine (6), and 7-methylguanine (6, 7). Resistance to mutagenesis has been attributed to the induction of high levels of an O6-alkylguanine methyltransferase (8–10).

Evidence for an adaptive response in mammals comes from the experiments of Montesano et al. (ref. 11; reviewed in ref. 12), showing that the livers of rats continuously exposed to low levels of dimethylnitosamine and then challenged with high doses of dimethylnitosamine, accumulate 7-methylguanine but not O6-methylguanine (O6MeGua) in their DNA. Of the several animal species used for such studies, only the rat shows an increase in methyltransferase.

In vitro, it has been shown that chronic treatment of Chinese hamster ovary (CHO) cells with nontoxic doses of MNNG renders the cells resistant to both killing and formation of sister chromatid exchanges upon further treatment with high toxic levels of MNNG or MNU (13). A single incubation of V79 Chinese hamster cells with a subtoxic dose of MNNG or MNU also modifies the cell sensitivity towards these agents (14).

In mammalian cells, 7-alkylguanine, 3-alkyladenine, and 3-alkylguanine are enzymatically excised through a DNA glycosylase pathway that creates an apurinic site (15–17), whereas O6MeGua is repaired by a transalkylase (18). Therefore, mammalian cells contain the potential proteins that could mimic the situation observed in adapted E. coli.

The aim of our work was to determine whether mammalian cells could be adapted for killing and mutagenesis by repeated treatment with low nontoxic concentrations of alkylating agents and whether these effects could be related to the type of damage induced by the drugs. We used simple alkylating agents [methyl methanesulfonate (MMS) and MNNG] that differ in their mutagenicity and give different spectra of methylated bases (19–21). The results show that adaptation to killing and mutagenesis are two independent processes and that adaptation to mutagenesis, in contrast to carcinogenesis, can be correlated with the amount of O6MeGua produced in cellular DNA during the adaptive treatment.

MATERIALS AND METHODS

Chemical Reagents. Dulbecco’s medium and sera were Gibco products. MNNG and MNU were purchased from Sigma. MMS was obtained from Aldrich. MNNG and MNU were dissolved in spectrophotometric grade acetone. Aliquots were placed in test tubes in the dark, and the acetone was evaporated under a stream of N2 gas. The tubes were kept at −20°C. Prior to use, the tubes were thawed, and the sample was diluted in Earle’s balanced salt solution (50 µg/ml). Solutions were used only once. [14C]MNNG (13.9 mCi/mmol; 1 Ci = 37 GBq) was obtained from New England Nuclear, and [14C]MMS (58 mCi/mmol), from Amersham.

Cell Culture. H4 cells, which are epithelial cells derived from a rat hepatoma (22) were obtained as a gift from J. B. Little (Harvard University). They were grown in Dulbecco’s medium supplemented with 5% fetal calf serum, 5% horse serum, penicillin (50 units/ml), and streptomycin (50 µg/ml) in humidified 5% CO2/95% air. The doubling time was about 14 hr. Cultures were periodically checked and found free of mycoplasma contamination. When H4 cells are cultured with daily medium changes, they reach a stable density-inhibited plateau in growth (22).

Mutagen Treatment and Survival Measurement. Mutagen pretreatment began 24 hr after seeding 5 × 105 cells in 25-cm² flasks (Falcon). Every 6 hr the medium was removed and the cells were incubated for 1 hr in 5 ml of serum-free medium that was either 0.27 µM in MNNG or 4.85 µM in MNU. In the case of MMS, every 12 hr the cells were incu-

Abbreviations: MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MNU, N-methyl-N-nitrosourea; MMS, methyl methanesulfonate; O6MeGua and N7MeGua, O6- and N7-methylguanine; 6SGua, 6-thioguanine.

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bated for 1 hr in 5 ml of serum-free medium that was 25 μM in MMS. At the end of each incubation, the cells were rinsed twice and reincubated in 5 ml of complete medium. These pretreatments were done over a 48-hr period and correspond to nine incubations with MNNG or MNU and to five incubations with MMS. Control cultures were treated in a similar manner except that the drug was omitted. Six hours after the final adapting dose, pretreated and control cells were incubated for 1 hr with the challenge doses of either MNNG, MNU, or MMS added to 5 ml of serum-free medium. Cells were in exponential phase of growth during the experiment. At the end of the challenging treatment, cells were rinsed twice and trypsinized, and then aliquots were plated in Petri dishes. Colonies were stained and counted after 12 days as described (23). The plating efficiency of control cells was between 80% and 90%.

**Mutation Frequency Determination.** Cells were cultured and pretreated as described above. Six hours after the final adaptive dose, control and pretreated cells were challenged for 1 hr with MNNG or MMS. They were rinsed, trypsinized, and immediately either plated for survival as described above or subcultured in fresh medium. After different expression times (1–9 days), the cells were plated in 100-mm Petri dishes (10² viable cells per dish) in Dulbecco's medium supplemented with 10% dialyzed fetal calf serum and 6-thioguanine (6SGua; 2.5 μg/ml). Colonies were counted after 15 days. Mutation frequency is expressed as the number of colonies in selective medium divided by (the number of cells plated × the viable fraction) (22).

**Analytical Methods.** Cells were grown in 75-cm² flasks, with or without pretreatment with low concentrations of alkylating agents under the conditions described above, and then exposed either to [¹⁴C]MMS (1 mM, 58 mCi/mmol) or to [¹⁴C]MNNG (1.7 μM, 13.9 mCi/mmol) in serum-free medium. After a 30-min incubation period, the cells were washed, trypsinized, and lysed by the addition of NaDodSO₄, and the cellular DNA was separated by centrifugation in CsCl density gradients as described (22). The DNA was recovered, dialyzed against potassium phosphate (1 mM, pH 7.6), and then hydrolyzed to purine bases and oligodeoxyribonucleotides as described by Frei et al. (24). DNA hydrolyzates were analyzed by using Waters HPLC equipment (25) as described by Frei et al. (24). The amount of DNA P was calculated as described by Medcalf and Lawley (26), and the amount of alkylated bases was determined by liquid scintillation counting.

Measurements of total acid-soluble thiols was as follows. Six hours after the end of the adaptive treatment, pretreated and control cells (3 × 10⁸ cells) were washed, harvested by trypsinization, centrifuged, and resuspended in 1 ml of 5% trichloroacetic acid. After centrifugation, aliquots of the supernatant were added to 1.4 ml of dithiobis(2-nitrobenzoic acid) (200 mg/ml in 0.2 M sodium phosphate buffer, pH 7.6) as described by Ellman (27). The absorption was measured at 410 nm, and the thiol concentration was obtained from a standard curve constructed by using reduced glutathione.

Protein and DNA concentrations were measured as described (22).

**RESULTS**

**Cell Survival.** The influence of a pretreatment with increasing MMS concentrations on the sensitivity of H4 cells to this drug was first determined. Every 12 hr the cells were incubated for 1 hr with different MMS doses. This pretreatment was carried out during a 48-hr period. Six hours later the cells were challenged with 4 mM MMS. The lack of toxicity was due to the pretreatment alone. Results show that the survival of challenged cells increased with the dose of alkylating agent used during the pretreatment (Fig. 1). The maximal enhancement of survival was observed with 25 μM MMS. Higher MMS concentrations were less effective in terms of adaptation because of decreases in the plating efficiency. Similar results were obtained with cells pretreated and challenged with MNNG. In the case of this compound, the pretreatment was done every 6 hr for 48 hr, and the optimal pretreatment concentration was found to be 0.27 μM (data not shown). Less increase of cell survival was observed when H4 cells were incubated fewer than five times with MMS or fewer than nine times with MNNG (data not shown). Therefore, the survival increase is a function of the drug concentration and of the number of incubations with the drug used during the pretreatment.

The survival of H4 cells pretreated with 25 μM MMS and then challenged with different MMS concentrations (Fig. 2A) was higher in pretreated than in control cells. When the cells were pretreated with MNNG and then challenged with different concentrations of this compound, an enhanced survival also was observed (Fig. 2B).

In order to investigate the crossreactivity with different alkylating agents, the survival of cells pretreated with MMS and then challenged with MNNG was measured. Results (Fig. 2C) also show an increased survival with this pretreatment. Other crossreactivity studies were performed with different alkylating agents. Table 1 summarizes the results of these experiments, expressed as the D₅₀ values. In all protocols of adaptation described, the pretreatment increased the cell survival, although this increment was slightly, but reproducibly, higher when the cells were pretreated and challenged with the same agent.

**FIG. 1.** Effect of varying the adaptive dose of MMS on the survival of H4 cells challenged with 4 mM MMS. Every 12 hr each culture was pretreated for 1 hr with a different MMS concentration. After 48 hr of adaptive treatment, the cells were challenged for 1 hr with 4 mM MMS and then plated for survival.

**Fig. 2.** Survival curves of H4 cells challenged with increasing doses of MMS or MNNG under nonadapted (●), or adapted (▲) conditions. (A) Cells pretreated with 25 μM MMS and challenged with MMS. (B) Cells pretreated with 0.27 μM MNNG and challenged with MNNG. (C) Cells pretreated with 25 μM MMS and challenged with MNNG.
Table 1. Crossreactivity of the adaptive response with different mutagens

<table>
<thead>
<tr>
<th>Adaptive drug</th>
<th>Challenge drug</th>
<th>$D_0$, $\mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>MMS</td>
<td>1100</td>
</tr>
<tr>
<td>MMS</td>
<td>MMS</td>
<td>2000</td>
</tr>
<tr>
<td>MNNG</td>
<td>MMS</td>
<td>1800</td>
</tr>
<tr>
<td>None</td>
<td>MNNG</td>
<td>8.5</td>
</tr>
<tr>
<td>MNNG</td>
<td>MNNG</td>
<td>16.6</td>
</tr>
<tr>
<td>MMS</td>
<td>MNNG</td>
<td>13.7</td>
</tr>
<tr>
<td>None</td>
<td>MNU</td>
<td>78</td>
</tr>
<tr>
<td>MNU</td>
<td>MNU</td>
<td>139</td>
</tr>
<tr>
<td>MNNG</td>
<td>MNU</td>
<td>125</td>
</tr>
</tbody>
</table>

The cells were pretreated with either MMS, MNNG, or MNU and then challenged with increasing concentrations of these compounds. $D_0$ values (drug concentration needed to reduce the surviving fraction by 1/e in the exponential portion of the survival curves) were obtained by regression analysis of data from three separate survival curves. Adaptation protocol and details are given in the text.

Different parameters that could modify the cell sensitivity towards MNNG were studied. No modification of the cell cycle occurred during the pretreatment period because in adapted cells the doubling time (14 ± 1 hr) and the mitotic index (1.5%) were identical to those determined in control cultures. During the pretreatment, there was no modification of the intracellular thiol level: the thiol concentration (expressed as $\mu g$ of glutathione per $\mu g$ of DNA) was 0.28 ± 0.02 and 0.29 ± 0.03 in adapted and control cells, respectively. Furthermore, alkylation of the cellular DNA was not modified by the pretreatment: after incubation of the cells with $[14C]\text{MNNG} (0.1 \text{ mM}, 11.3 \text{ mCi/mol})$ for 30 min, the same amount of radioactivity corresponding to 2.86 ± 0.05 pmol of MNNG per $\mu g$ of DNA was incorporated in both control and adapted cells.

**Mutation Frequency.** The influence of pretreatment with alkylating agents on cell mutagenesis was tested by measuring the appearance of 6SGua-resistant cells. When H4 cells were adapted with MNNG and then challenged with different concentrations of the same agent, the mutation frequency, measured 5 days after the treatment, was markedly reduced compared to control cultures (Fig. 3A).

Because this low mutation frequency in adapted cells could be due to a modification of the expression time in the pretreated cultures, the number of mutants was determined at different times after the incubation with each challenge dose of mutagen (1–9 days). In both control and pretreated cells, the optimal expression time was found to be 5 days. Therefore, this hypothesis cannot account for the decrease of mutation frequency in adapted cells.

When the cells were pretreated with MMS and then challenged with the same compound, the mutation frequency was not significantly modified under our experimental conditions (Fig. 3B). The same result was obtained in a cross-experiment, with cells pretreated with low MMS doses and then challenged with MNNG (Fig. 3C): adapted and non-adapted cells produced an equal number of 6SGua-resistant mutants. Therefore, a pretreatment with MMS enhances the survival of cells challenged with either MMS or MNNG as shown in Fig. 2 but is unable to modify the mutation frequency induced by these two compounds.

**Kinetics of O6MeGua Removal.** As mutagenesis has been related in a number of experiments to the persistence of O6MeGua in cellular DNA, experiments were designed to measure the mutation frequency in control and adapted cells as a function of the kinetics of O6MeGua removal. In order to avoid dilution of bases by DNA synthesis, an appropriate number of cells ($5 \times 10^5$ cells in 75-cm² flasks) was seeded that allowed the cultures to reach confluency at the end of the pretreatment period. Under these conditions, the adaptive response was similar to that described above. Control cells and cells pretreated with either MMS or MNNG were incubated for 30 min with $[14C]\text{MNNG} (0.27 \text{ mM})$. The cells were washed and harvested after 0, 2, 3.5, or 24 hr of subsequent incubation. The cellular DNA was then purified and hydrolyzed, and the O6MeGua was separated by HPLC and determined.

Fig. 4 shows the kinetics of O6MeGua removal. The initial amount of induced O6MeGua had the same value in control cells and in cells with and without adaptation with MMS or MNNG. In control and MMS-pretreated cells, O6MeGua was removed with the same slow biphasic kinetics; during the early postincubation times, about 1.7 $\mu$mol of O6MeGua per mol of DNA $P$ was removed per hr, whereas later the rate of removal decreased to 0.23 $\mu$mol/hr. In MNNG-pretreated cells, O6MeGua was also removed with two-compo-
rient kinetics. However, this lesion was removed with a much higher efficiency than in control or in MMS-adapted cells. In this case, the rate of removal was about 5 μmol of \( O^\circ MeGua \) per mol of DNA \( P \) per hr, and no \( O^\circ MeGua \) was detectable 24 hr after the challenging treatment. The kinetics of \( N^\circ -methylguanine (N^\circ MeGua) \) removal was determined also and found to be at variance with that observed for \( O^\circ MeGua \) because \( N^\circ MeGua \) was removed at the same rate in control and in MMS- or MNNNG-pretreated cells, with a half-life of \( \approx 25 \) hr (data not shown).

**Comparative Alkylation of Cellular DNA by MMS or MNNNG.** In order to explain the different cell responses observed with MMS and MNNNG with respect to mutation frequency and kinetics of \( O^\circ MeGua \) removal, the amount of \( O^\circ MeGua \) induced in \( H_t \) cells by these two agents was measured. \( N^\circ MeGua \) also was measured as an internal control. \( H_t \) cells (nonpretreated) were incubated for 1 hr with equitoxic doses of \( [14C]MMS \) (1 mM) or \( [14C]MNNNG \) (1.7 μM). The cellular DNA was purified and hydrolyzed, and then the amounts of \( O^\circ MeGua \) and \( N^\circ MeGua \) residues were determined by HPLC analysis. Equitoxic concentrations of MMS or MNNNG yielded about the same amount of \( O^\circ MeGua \) and induced the same number of 6SGua-resistant mutants (Table 2). MMS induced about 25-fold more \( N^\circ MeGua \) than did MNNNG, suggesting that this lesion is not involved in cell killing. These results also show that a 588-fold higher MMS than MNNNG concentration is required to induce the same amount of \( O^\circ MeGua \).

The concentrations of MMS (25 μM) and MNNNG (0.27 μM) used during the pretreatment did not allow accurate determination of the amounts of \( O^\circ MeGua \) by analysis of cellular DNA. By assuming that these amounts are proportional to the concentration of the alkylation agent, they can be extrapolated from the data reported in Table 2: in order to obtain with MMS the same amount of \( O^\circ MeGua \) as that formed with 0.27 μM MNNNG in the cellular DNA, one needs 158 μM MMS (0.27 × 588). However, as shown in Fig. 1, this MMS concentration is much too toxic and does not induce adaptive response in the cells.

**DISCUSSION**

When \( H_t \) cells are exposed to low (nontoxic) concentrations of alkylation agents, they become less sensitive to the toxic effect of these compounds. We observed this phenomenon with MMS, MNNNG, and MNU, and crossreactivity of the adaptive response towards cell survival was also observed with these drugs.

It has been shown that the cell sensitivity towards MNNNG could fluctuate throughout the cell cycle (28) and that nonproliferating cells were more sensitive to MMS than exponentially growing cells, the increased sensitivity arising from the loss of the shoulder that is a characteristic of the survival curves obtained with this drug (29). However, the survival enhancement observed in adapted cells cannot be related to such modifications, as the cell cycle was not modified during the pretreatment period under our experimental conditions. It also has been shown that MNNNG is activated by thiol groups (30). Therefore, a modification of the intracellular thiol level during the pretreatment could explain the enhanced cell survival observed with this compound. This hypothesis was ruled out, as the total thiol level was not modified in adapted cells. It also was determined that the alkylation of cellular DNA by MNNNG was not modified after the pretreatment period.

An adaptive response to the killing effect of alkylation agents (MNNNG and MNU) has been reported in CHO cells (13) and in V79 cells (14) with different adaptive protocols. Therefore, adaptation to the toxic effect of alkylation agents occurs in different cell lines, but the survival enhancement varies among the cell lines studied. The adaptive response in mammalian cells is of lesser magnitude than that reported to be in bacteria, although it should be recalled that in *E. coli*, adaptation is less effective in the K12 strain than in B/r (31).

However, controversy exists about the adaptive response of mammalian cells to mutagenicity. After 48 hr of chronic pretreatment with MNU, the mutagenicity of CHO cells is not modified when the cells are challenged with the same agent (32). Pretreatment with MNU increases the cell survival but does not modify the mutagenicity of V79 cells (33). Opposite results were obtained by Kaina *et al.* (14), who showed that a single exposure of V79 cells to a subtoxic dose of MNU or MNNNG renders the cells resistant to the mutagenic effect of these agents given 6 hr later.

Under our experimental conditions, pretreatment with MNNNG renders the cells resistant to the mutagenic effects of high concentrations of this compound. However, pretreatment with MMS is unable to induce mutagenic resistance in cells challenged with either MMS or MNNNG. The crossreactivity observed for cell survival does not exist for mutagenicity. This suggests that adaptive responses to killing and mutagenesis are at least partially independent processes.

Among the different lesions induced in cellular DNA by alkylation agents, at least six, including \( O^\circ -alkylguanine \) are mutagenic (20). In the case of \( O^\circ -alkylguanine \), the lesion is repaired by an alkyl transferase (18, 34), which is present in limited amounts in the cells (9). A possible pathway leading to mutation resistance could be a more efficient \( O^\circ MeGua \) removal in adapted cells. Although mammalian cells differ in their ability to repair \( O^\circ MeGua \) (35, 36), it has been shown that in vivo rat liver cells were able to remove this lesion (37). Under our experimental conditions, cultured rat hepatoma cells remove \( O^\circ MeGua \), and the results show a correlation between the efficiency of \( O^\circ MeGua \) removal and the decrease of mutagenicity in MNNNG-pretreated cells. Furthermore, the MMS-pretreatment, which is not effective to adapt to mutagenesis, does not modify the disappearance of \( O^\circ MeGua \) in \( H_t \) cells.

As reported for other cell lines (36), the rate of \( O^\circ MeGua \) removal in control or MMS-adapted cells decreases with time. This suggests that in both cases the protein involved is consumed by the reaction and, therefore, is present in a limited amount in the cells. Because the removal of \( O^\circ MeGua \) is faster and more efficient in MNNNG-pretreated cells, these cells could contain more \( O^\circ MeGua \) transferase than do control cells. An increase of the amount of acceptor protein after multiple exposures to MNNNG occurs also in HeLa cells (38), but this result has not been correlated to the mutation rate in the cells.

\( N^\circ MeGua \) was removed at the same rate in control and in adapted cells, with a half-life of \( \approx 25 \) hr, which is in agreement with data obtained with different cell lines (26), and

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**Table 2.** Comparison of cell survival, mutation frequency, and amounts of \( O^\circ MeGua \) and \( N^\circ MeGua \) induced by MMS or MNNNG in \( H_t \) cells

<table>
<thead>
<tr>
<th>Alkylation agent</th>
<th>( O^\circ MeGua, \mu\text{mol/mol of Gua} )</th>
<th>( N^\circ MeGua, \mu\text{mol/mol of Gua} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Variable} )</td>
<td>( \text{MMS} )</td>
<td>( \text{MNNNG} )</td>
</tr>
<tr>
<td>Concentration, ( \mu\text{M} )</td>
<td>1000</td>
<td>1.7</td>
</tr>
<tr>
<td>Cell survival, %</td>
<td>70</td>
<td>75</td>
</tr>
<tr>
<td>Mutation frequency per 10(^5) survivors</td>
<td>9 ± 2</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>( O^\circ MeGua )</td>
<td>4.5</td>
<td>5</td>
</tr>
<tr>
<td>( N^\circ MeGua )</td>
<td>1660</td>
<td>67</td>
</tr>
</tbody>
</table>

Control cells were incubated for 1 hr with \( [14C]MMS \) (1 mM) or \( [14C]MNNNG \) (1.7 μM), and the amounts of \( O^\circ MeGua \) and \( N^\circ MeGua \) were determined. Parallel cultures were incubated with nonradioactive drugs and then seeded for survival and mutagenicity determinations.
rules out the possibility that O6MeGua loss in adapted cells could be due to cell death or division. The pretreatment by MNNG results in a 3-fold increase in the rate of O6MeGua removal, although it renders the cells resistant to the mutagenic effect of this compound under our experimental conditions. This discrepancy could be related to the involvement of other lesions in the adaptive process—for instance, O4-methylthymine, which is removed by a transalkylase in E. coli (unpublished data).

Because different responses were obtained with MMS and MNNG as adaptive drugs, the amounts of two derivatives produced by these compounds, O6MeGua and N4MeGua, were determined in order to ascertain whether either derivative could be correlated with the two biological end points. At equitoxic concentrations, MMS and MNNG produce an equal number of mutants and the same amount of O6MeGua, but MMS produces about 25-fold more N4MeGua. Similar results were obtained in hamster embryo fibroblasts (39). This result suggests that N4MeGua is implicated neither in cell killing nor in adaptive response. However, these equitoxic concentrations differ greatly, the MMS/MNNG concentration ratio being about 588.

If we assume that this ratio is also true when the cells are treated with low concentrations of alkylating agents (40), this means that the MMS concentration of 25 μM, which adapts the cell for survival but not for mutagenesis, induces much less O6MeGua than does the MNNG dose (0.27 μM) used during the pretreatment. However, higher MMS concentrations cannot be used during the pretreatment because of the toxicity of this compound. Therefore, if O6MeGua is involved in inducing killing resistance, it is probably not the only damage involved in this process. However, O6MeGua appears to be essential to induce mutagenesis resistance, and adaptation to mutagenesis is probably related to the amount of O6MeGua produced during the adaptive period, although the role of other alkylated bases cannot be excluded.

Variations in the amount of O6MeGua induced in the pretreated cells are a probable explanation for the failure of other investigators to find adaptation to mutagenesis. Adaptive treatments might produce enough damage to induce killing resistance but not enough O6MeGua to render the cells resistant to mutagenesis. It should be recalled that the extent of alkylation varies with the nature of the drug (19) and the drug concentration (40) used during the pretreatment. In the case of MNNG, the intracellular thiol concentration is also critical in the generation of the methylation species (30).

It should be pointed out that most of the drugs used during cancer therapy are or are metabolized to alkylating agents. The appearance of an adaptive response during these treatments, which are pursued for long periods, cannot be excluded. Therefore, according to the drug and its methylation properties, adaptation could occur for survival or for mutagenesis, thus modifying the efficiency of the treatment or the appearance of iatrogenic diseases.

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