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DECREASED EXCISION OF O^6-METHYLGUANINE AND N^7-METHYLGUANINE DURING THE S PHASE IN 10T1/2 CELLS

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SUMMARY: The excision of N^7-methylguanine (N^7-meGua) and O^6-methylguanine (O^6-meGua) lesions in DNA caused by treatment of 10T1/2 cells with N-methyl-N'-nitro-N-nitrosoguanidine was evaluated as cells synchronously traversed the pre-S and S phases of the cell cycle. Proliferation of cells was arrested by growth to confluence, then cells were treated with MNNG and released into a synchronous cell cycle by replating at lower density. The frequency of the two methylated guanines (methylated guanines/10^6 guanines) was determined at the time of replating, immediately prior to the onset of S phase and at the conclusion of S phase. During the pre-S interval N^7-meGua and O^6-meGua were lost at rates consistant with the reported biological half-lives of 26-28 hr and 20-21 hr, respectively. In contrast, when the reduction in frequency of methylated guanines was determined for the S phase it was found that the apparent decrease could be explained by the increased DNA content of the cultures resulting from DNA replication.

Ionizing radiation and chemical carcinogens cause various types of damage to DNA that are recognized by mammalian cells and are either removed or circumvented by DNA repair processes. The principal cellular mechanisms for correcting DNA damage collectively have been termed excision repair. Whereas specific enzymes appear to be responsible for the initial removal of particular types of DNA damage, the process of repair ultimately yields an excised segment of DNA the resynthesis of which may occur via a common mechanism (1). Enzymatic excision and resynthesis at sites of DNA damage is well documented in nonproliferating cells, in G_1 or G_0 phases of the cell cycle, and even during logarithmic growth of cultures (2). There have been few studies, however, to evaluate excision repair during the S phase. Repair activity induced by ultraviolet irradiation (UV) or 4-nitroquinoline-oxide (4-NQO) has been reported to occur in S phase cells, based upon observations of radiolabel incorporated into parental DNA (3,4). How-

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ever, incorporation of radiolabel into parental DNA in S phase cells generally represents less than 1% of the background level of replicative DNA synthesis, so that it is difficult to precisely distinguish repair activity from replicative activity during S phase (5). To evaluate DNA repair during S phase while avoiding the uncertainties in attempting to detect reparative DNA synthesis coincidental with DNA replication, we have measured directly the loss of specific carcinogen adducts from DNA during the S phase of a synchronous cell cycle (6).

MATERIALS AND METHODS

All experiments were conducted with C3H 10T1/2 mouse cells isolated in Dr. C. Heidelberger's laboratory and obtained from Dr. John Little of Harvard University. The 10T1/2 cells routinely were maintained as described by Reznikoff, et al (7). For synchronization of the progression through the cell cycle, the cultures were initially allowed to grow to confluence at which time cell proliferation ceased. While cell proliferation was arrested at confluence, the culture medium was replaced with Hank's balanced salt solution (BSS) containing 2 μg/ml of ^{14}C-N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; 0.34 mCi/mmole) and cultures were incubated for 30 min at 37° in a humidified atmosphere containing 5% CO_2. The cells then were rinsed free of MNNG with BSS, trypsinized to remove them from the plates, and combined. At this time DNA was purified from one-third of the cells (as described below), while the remaining cells were replated at 1 x 10^6 cells/100 mm plate in Eagles Basal Medium (BME) (Gibco) supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (50 mg/l) (Schering). At 17 hr and at 34 hr after replating, half of the replated cells were dissociated by trypsinization, rinsed free of medium with BSS, and pelleted by centrifugation. To isolate DNA from these and the initial aliquot, cell pellets were resuspended in 10 mM EDTA, 10 mM Tris-HCl, pH 7.8, containing 1.5 mg/ml Proteinase K and deproteinized during a 30 min incubation at 37°C. Cesium chloride was added directly to the hydrolysate to give a final refractive index of 1.4000. The DNA preparations were centrifuged to equilibrium at 35,000 rpm for 66 hr. The fractions containing DNA were pooled, dialyzed exhaustively, and reduced in volume. The final DNA concentration was then determined from the A_{258}. The purified DNA solution was made 0.1 N in HCl, and the purine bases were released by hydrolysis at 37°C for 20 hr. The free purine bases were chromatographically separated on a Sephadex G-10 column (90-1.5 cm), eluted isocratically with 50 mM ammonium formate, pH 6.8.

The cellular progression through the cell cycle was followed by pulse labelling cells with ^{3}H-Thd at intervals following release from growth arrest. Aliquots of cells exposed to 2 μg/ml of MNNG and then released from growth arrest were incubated with a 30 min pulse of ^{3}H-Thd (1 μCi/ml) every two hours. The cells were fixed with 5% TCA, then harvested, and the radiolabel incorporated into the DNA was quantitated.

RESULTS

Cells whose proliferation was arrested by growth to confluence, were exposed to MNNG (2 μg/ml) and subsequently replated at lower density. Following replating, cells reach the G_1/S transition point in 17-18 hr and complete S

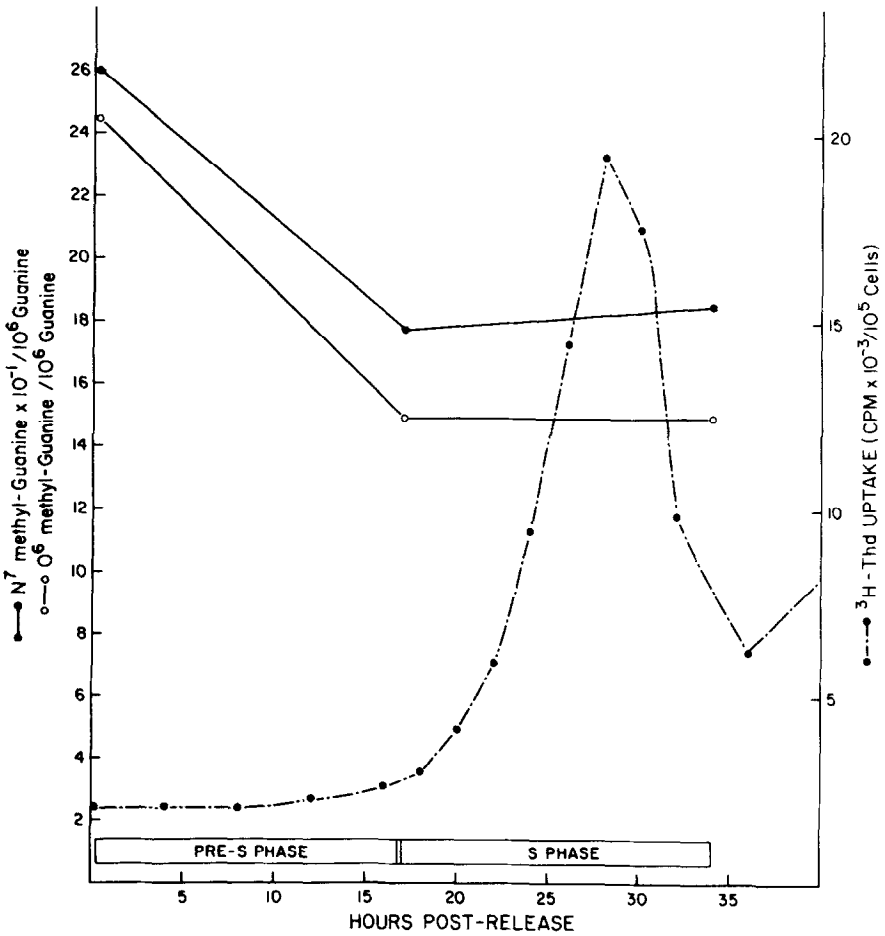

Figure 1 Thymidine labelling and frequencies of methylguanine lesions in DNA as a function of time after release of 10T1/2 cells from confluence arrest. After cessation of cell proliferation following growth of 10T1/2 cells to confluence, cultured cells were treated with MNNG, then released from confluence arrest by replating, and periodically aliquots of cells were pluse labelled with ^{3}H-Thd (●---●) as described in Materials and Methods. The frequencies of O^{6}-meGua (O—O) and N^{7}-meGua (●—●) in the parental DNA exposed to ^{14}C-MNNG (Table 1) also was determined for cells at the time of release from confluence (0 hr), immediately prior to the S phase (17 hr) and at the end of S phase (34 hr).

phase by 34-35 hr (FIGURE 1). The quantity of DNA recovered from cultured cells immediately after replating (T_0) was 420 µg and at 17 hr (T_{17}) was 351 µg indicating that little or no DNA replication had occurred during this interval (TABLE 1). Exposure of arrested cells to MNNG produced an average of 24 O^{6}-methylguanine (O^{6}-meGua) residues per 10^{6} guanine residues and 263 N^{7}-methylguanine (N^{7}-meGua) residues per 10^{6} guanine residues in the parental DNA. At

TABLE 1

Frequency Of O^6-meGua And N^7-meGua Lesions In DNA Following Exposure Of 10T1/2 Cells To ^{14}C-MNNG

Hours After MNNG Exposure	Total DNA Recovered (µg DNA/2x10^7 cells)	Lesion Frequency in Total DNA		Lesion Frequency in Parental DNA	
		N^7-meGua/10^6Gua	O^6-meGua/10^6Gua	N^7-meGua/10^6Gua	O^6-meGua/10^6Gua
0	420	263	24	263	24
17	351	181	15	181	15
34	610	100	8	186	15

Cells were exposed to ^{14}C-MNNG in confluence arrest and released by replating at a lower density. The frequency of O^6-meGua and N^7-meGua was determined at the time of release, at the beginning of the S phase (17 hrs), and at the conclusion of the S phase (34 hrs).

the G_1/S border 17 hr after replating, the frequency of O^6-meGua adducts had been reduced from 24 to 15 per 10^6 guanine residues. This 36% decrease occurred during the pre-S interval, a period during which there was no apparent increase in the cellular DNA content. The frequency of N^7-meGua residues changed from 263 to 181 per 10^6 guanine residues, a 31% reduction between T_0 and T_{17}.

An 87% greater quantity of cellular DNA was recovered from cultures at 34 hr after replating, following completion of the synchronous S phase. O^6-meGua adducts decreased from 15/10^6 guanine residues at 17 hr to 8/10^6 guanine residues at 34 hr, a decrease of 47%. N^7-meGua substituents were reduced from 180/10^6 guanine residues at 17 hr to 100 N^7-meGua adducts per 10^6 guanine residues at 34 hr, a reduction of 44%. The observed reduction in O^6-meGua and N^7-meGua residues per 10^6 guanine residues represents the values uncorrected for dilution by the newly synthesized DNA which nearly doubled the total DNA content. The 87% increase in total DNA, and therefore in total guanine residues, means that only 53% of the total recovered guanine residues, or total DNA was originally in the parental DNA exposed to MNNG at T_0. Consequently, when the data are recalculated for binding to DNA recovered at 34 hr with respect to the parental DNA existing at that time (T_0), the true frequencies of O^6-meGua or N^7-meGua residues in the parental DNA are given. The true frequency of methylated guanines in the parental DNA at T_{34} is 15 O^6-meGua/10^6 guanine residues, and 186 N^7-meGua/10^6 guanine residues. These corrected values are indistinguishable from those observed at 17 hr, considering the experimental error inherent in the study. Consequently, loss of these DNA lesions does not appear to occur during S phase.

DISCUSSION

The N^7 position of guanine is the principal site of DNA methylation by MNNG, but alkylation of oxygen substituents of the DNA bases, such as the O^6 position of guanine, is believed to be better correlated with carcinogenicity

and toxicity (8). For these reasons we studied the removal of these two DNA lesions during synchronous passage of 10T1/2 cells through the cell cycle. The extent of initial alkylation of the N^7- and O6- positions of guanine was evaluated as the number of methylated guanines per 10^6 guanine bases in the parental DNA exposed to MNNG at T_0. Normalizing to the fraction of methylated guanines removes the potential for error introduced by the decrease in total DNA associated with the modest cytotoxicity of this treatment with MNNG. Furthermore, the use of synchronized cultures minimizes the error in estimates of excision repair that could result from the dilution of DNA lesions by replication of DNA. By correcting for the increase in cellular DNA at the end of S phase, the frequency of N^7-meGua and O^6-meGua residues remaining in the parental DNA exposed to MNNG at T_0 can be determined.

N^7-meGua and O^6-meGua residues were removed effectively during the pre-S interval (0-17 hr). The 36% loss of O^6-meGua adducts during the 17 hr pre-S interval is comparable to that predicted from the 21-24 hr biological half-life reported in other systems (9,10). The 31% loss of N^7-meGua adducts observed in the pre-S interval is consistent with reports of a 24-26 hr half-life (11-13), but differs from studies which indicate a much longer half-life in the rat in vivo (10,14). It has been suggested that in the latter instances, the N^7-meGua lesions are slowly lost by a non-enzymatic process. In contrast, the more rapid rate of removal, as we have observed, suggests an enzyme-mediated removal in addition to spontaneous depurination. Further studies are necessary for the direct determination of the rate of removal (or alternatively, the half-lives) of these adducts within various phases of the cell cycle in 10T1/2 cells.

Decreases in the ratios of methylated bases were observed for N^7-meGua (44%) and O^6-meGua (47%) residues during the S phase interval (17-34 hr). During this interval, however, the total DNA content increased by 87%. Consequently, the decreased ratios of alkylated guanines per 10^6 total guanines can be attributed, within the precision of the experiment, to dilution of alkyl-

ated DNA with DNA newly synthesized during S phase. The obserd absence of excision of N^7-meGua and O^6-meGua adducts during the S phase of synchronous 10T1/2 cells is not necessarily in conflict with earlier reports of repair of 4-NQO or UV damage in other S phase cells. Methylated nitrogenous bases are removed from DNA by N-glycosylases via the base excision repair pathway. In contrast, UV or 4-NQO lesions are excised with a substantial length of DNA by nucleotide excision repair (1). Since excision of lesions produced by 4-NQO and UV have been shown to be inhibited in the same class of xeroderma pigmentosum cells, it is assumed that both types of damage are repaired by the same enzymatic system. These same cells, however, have a normal capacity for repair of DNA lesions caused by MNNG (15). The fact that the present study shows an absence of removal of damaged DNA during the S phase and previous reports describe a continuing reparative DNA synthesis constitutes another substantial difference. Excision of damage could be inhibited while the resynthesis of DNA in previously created single-stranded gaps could proceed during the S phase.

The process of DNA excision repair, both base repair and nucleotide repair, and the steps of excision of damaged bases and reparative DNA synthesis require further study to determine their activities during the S phase. This information would provide a better understanding of whether DNA repair processes have a protective function during DNA replication. In turn this knowledge would offer greater insight into the role of excision repair of radiation or chemical induced DNA damage in carcinogenesis.

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