Dose- and Cell Cycle-dependent O<sup>6</sup>-Methylguanine Elimination from DNA in Regenerating Rat Liver after [<sup>14</sup>C]Dimethylnitrosamine Injection

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ABSTRACT

To study relations between increased transformation sensitivity during DNA synthesis and carcinogen-induced molecular DNA alterations in regenerating liver, the kinetics of formation and the persistence of alkylated DNA bases were determined 10 to 240 min after a single [<sup>14</sup>C]dimethylnitrosamine (DMN) injection (1.43 and 4.0 mg/kg) during G<sub>1</sub>, 12 h after partial hepatectomy and in synchronized S phase (4 hr after continuous infusion of hydroxyurea after partial hepatectomy; about 80% hepatocytes in DNA synthesis).

At 120 min after administration of DMN, the molar fraction of 7-methylguanine reached a maximum which was proportional to the injected DMN dose in G<sub>1</sub> but lower by about 25% in S-phase cells. During G<sub>1</sub>, the molar fraction of O<sup>6</sup>-methylguanine revealed the same formation kinetics as 7-methylguanine; but in S-phase cells, it was reduced by 60% after 1.43 mg/kg and by 35% after 4.0 mg/kg. 3-Methyladenine reached its maximum earlier in S phase than in G<sub>1</sub>, with a faster decline in S than in G<sub>1</sub>.

As soon as 10 min after DMN was given, the O<sup>6</sup>-methylguanine/7-methylguanine ratio was significantly lower in S-phase cells than in G<sub>1</sub> cells, with a rapid further decrease during 30 min shortly after carcinogen exposure during DNA synthesis. This rapid repair was less expressed after 4.0 mg/kg, indicating an exhaustion of this process after higher carcinogen doses.

Cell cycle-dependent differences of the kinetics of O<sup>6</sup>-methylguanine and 3-methyladenine in DNA in vivo confirm earlier observations in vitro that these adducts are released by different enzymatic mechanisms that the activity of which appears to be enhanced during DNA synthesis in rat liver (Pegg et al., Biochem. J., 197: 195–201, 1981; Gombaret al., Carcinogenesis, 2: 595–599, 1981).

The faster elimination of the promutagenic O<sup>6</sup>-methylguanine during DNA synthesis could represent an important but, because of its saturation at higher carcinogen dosages, not sufficiently effective mechanism to prevent proliferating cells from initiation during the most critical period of the cell cycle.

INTRODUCTION

The proliferative state of a target cell population appears to be critical for initiation of malignant transformation (9, 38, 43). Rat liver is a frequently used model for studying correlations between proliferation and initiation of carcinogenesis (8, 38, 56).

Injection of a single dose of a chemical carcinogen usually fails to induce tumors in the intact liver of adult rats (8). After partial hepatectomy, however, low amounts of various carcinogens given as a single dose are capable of inducing liver tumors (8, 38). Because of a peculiar heterogeneity of the proliferative response of hepatocytes after partial hepatectomy in various zones of the liver lobule (37, 41, 42), it is difficult to relate a specific cell cycle period to the observed increment of tumor incidence after a carcinogen dose following partial hepatectomy.

By means of a continuous infusion of HU, it is possible to accumulate the total growth fraction of hepatocytes near the G<sub>1</sub>-S boundary and to obtain an almost pure S-phase compartment comprising about 80% of all hepatocytes after release from HU block (39). With this model, it is possible to compare the tumor-inducing capacity of DMN and the pattern of DNA base alkylation in selective compartments of the cell cycle (40).

Alkylating N-nitroso compounds exert carcinogenic effects in all probability by interaction of electrophilic ultimate carcinogenic reactants with nucleophilic sites of informational macromolecules of the cell (28). Although the exact mechanisms of the initiation process are far from being understood, it is an attractive hypothesis that DNA base alkylation at sites involved in hydrogen bonding may belong to the critical steps of initiation. Besides other alkylations products (O<sup>2</sup>-thymine and O<sup>2</sup>-cytosine (48)), O<sup>6</sup>-alkylguanine in particular has been accused to be a promutagenic base alteration (22) giving rise to base mispairing (Refs. 1, 10, and 27; see also Refs. 23 and 50). The presence and persistence of O<sup>6</sup>-alkylguanine have been shown to be closely related to the tumor-inducing capability of carcinogenic N-nitroso compounds in various organs (12, 16, 30).

Using the HU-synchronized regenerating rat liver system in vivo, it has been proved that the specific tumor-inducing effect of a single dose of DMN during DNA synthesis was not combined with an increased formation of the putatively promutagenic O<sup>6</sup>-methylguanine; on the contrary, the O<sup>6</sup>-methylguanine/7-methylguanine ratio was significantly lower during the initiation-sensitive S phase as compared with G<sub>0</sub> or G<sub>1</sub> (40). It will be demonstrated that the kinetics of O<sup>6</sup>-methylguanine formation and removal discloses a cell cycle-dependent pattern, suggesting a protective mechanism of limited effectiveness during the critical phase of DNA synthesis. The results confirm and extend earlier observations in vitro about the increased activity of an O<sup>6</sup>-methylguanine- and 3-methyladenine-eliminating principle in extracts of regenerating rat liver (11, 36).

MATERIALS AND METHODS

Treatment of Animals. Male Wistar AF/Hann rats (210 to 230 g) bred in our animal house were used for the experiments. They were...
kept on Altromin-R standard diet (Lage, Lippe, West Germany) and water ad libitum. Two-thirds hepatectomies (14) were performed under light ether anesthesia. To accumulate hepatocytes at the G\textsubscript{1}-S boundary, the rats received a continuous i.v. infusion of HU (highest purity; Serva Feinbiochemica, Heidelberg, West Germany) from 14 to 39 hr after partial hepatectomy as described in previous papers (39, 40). By this procedure, the total growth fraction is arrested at the G\textsubscript{1}-S boundary and proceeds, after HU block, through DNA synthesis (39), giving by these means a possible way to evaluate DNA base alkylation in a relatively homogeneous compartment of DNA-synthesizing hepatocytes.

Determination of Kinetics of DNA Base Alkylation after DMN Injection. \(^{14}\text{C}\)DMN was injected i.p. at a dosage of 1.43 or 4.0 mg/kg body weight (New England Nuclear; final specific activity, 10.3 and 3.7 mCi/mmol; 0.2 \(\mu\)Ci/g body weight). Alkylation of DNA bases and repair kinetics were evaluated during G\textsubscript{1} (\(^{14}\text{C}\)DMN 12 hr after partial hepatectomy), and during HU-synchronized S (43 hr after partial hepatectomy, 4 hr after HU infusion). The rats were sacrificed at 10, 20, 30, 60, 120, or 240 min after \(^{14}\text{C}\)DMN injection, respectively. In order to improve the radioactivity measurements of DNA bases for the short intervals (10, 20, and 30 min) after \(^{14}\text{C}\)DMN injection, the total amount of administered radioactivity was increased to 0.4 \(\mu\)Ci/g body weight. The specific activity of the injected material was adjusted accordingly. After sacrifice, the liver tissue of 3 rats of each group was pooled and kept at 4°C during the DNA extraction procedure. Most experiments were repeated at least twice.

Extraction of DNA and Analysis of Alkylation Products. Pooled liver tissue was maintained at 4°C for a maximum of 5 min until homogenization in an ice bath. The procedure of DNA extraction originally reported by Kirby and modified by Swann and Magee (54), described in detail in a previous paper (40), has been used in the present experiments. Quantitative determination of DNA bases was confined to the purine bases adenine and guanine and their alkylated derivatives. DNA was subjected to mild acid hydrolysis in a solution of 1.5 mg DNA per ml of 0.1 N HCl for 16 to 20 hr at 37°C (19). The subsequent separation of DNA bases and methylated products using Sephadex G-10 radiochromatography was performed as already described (40).

Molar fractions of the methylated purines were calculated from the radiochromatograms according to the method of Swann and Magee (54), assuming the identity of the specific activity of the methylated base and the injected \(^{14}\text{C}\)DMN. The same method of calculation was used for the determination of the metabolically labeled guanine and adenine due to incorporation of radioactive 1-carbon precursors.

RESULTS

As early as 10 min after a single i.p. injection of \(^{14}\text{C}\)DMN at a dosage of 1.43 or 4.0 mg/kg, sufficiently labeled fractions of DNA base hydrolysates were recovered from Sephadex G-10 radiochromatograms to permit a quantitative evaluation of alkylation products (Tables 1 and 2).

Comparison of the radiochromatograms after \(^{14}\text{C}\)DMN (1.43 mg/kg) given during G\textsubscript{1} and synchronized S phase reveals typical differences (Chart 1). During S phase, a small amount of \(^{14}\text{C}\)guanine and adenine formed from the labeled 1-carbon pool by synthesis is found after only 10 min of exposure with highly increased values after 240 min (Chart 1, C and D). After exposure during G\textsubscript{1}, no metabolic incorporation is observed during the first 10 min, but there is a slight increment of \(^{14}\text{C}\)guanine and pyrimidines after 240 min, i.e., 16 hr after partial hepatectomy. Those few cells which have already entered DNA synthesis at this time explain this limited metabolic incorporation of the G\textsubscript{1} compartment (Chart 1, A and B). Besides these labeled fractions, the main radioactivity in both experimental groups belongs to the formation of 7-methylguanine, O\(^{6}\)-methylguanine, and 3-methyladenine. The overall pattern appears similar in both groups at 10 min (Chart 1, A and C) but is apparently different at 240 min, especially with regard to the amount of O\(^{6}\)-methylguanine present (Chart 1, B and D). Formation of 7-methylguanine as a function of time after a single \(^{14}\text{C}\)DMN injection attains the highest values after exposure during G\textsubscript{1} with both DMN dosages used (Charts 2 and 3). A remarkable identity of the initial alkylation values is observed in G\textsubscript{1} cells 10 min after 1.43 and 4.0 mg/kg, respectively. This indicates a relatively low initial rate of DMN metabolism for the larger dose. The maximum is reached at about 120 min, at which time the alkylation shows an approximate proportionality to the injected dose. A reduction of a molar fraction of 7-methylguanine is seen thereafter. This decline of 7-methylguanine at late intervals after DMN appears to be more pronounced during G\textsubscript{1} than during S phase. Low metabolic \(^{14}\text{C}\) incorporation from the 1-carbon pool into guanine at 240 min in G\textsubscript{1}, liver (Tables 1 and 2) suggests that this reduction of the molar fraction of 7-methylguanine is not due to DNA replication

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Interval (min)</th>
<th>7-Methylguanine</th>
<th>O(^{6})-Methylguanine</th>
<th>3-Methyladenine</th>
<th>[^{14}\text{C}]Guanine</th>
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<td>1.07</td>
<td>0.22</td>
<td>2.36</td>
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\(\text{a}\) Between DMN injection and sacrifice of rats. 
\(\text{b}\) Means of 1 to 3 experiments, each with 3 rats; molar fraction \(\times 10^6\).  
\(\text{c}\) Twelve hr after partial hepatectomy.  
\(\text{d}\) Four hr after the end of a continuous HU infusion after partial hepatectomy.
but that it might represent enzymatic excision of this DNA adduct (5, 24, 26, 36).

Under the conditions of synchronized DNA synthesis, the initial increase of 7-methylguanine is similar to the situation in G1, but with a steeper increment at early intervals after 4.0 mg/kg (Chart 3). However, the continued DNA synthesis as evident from the high rates of metabolic incorporation into guanine from the 14C-labeled 1-carbon pool (Tables 1 and 2) leads to the relative diminution of the molar fraction of 7-methylguanine with respect to the parent base guanine as early as 60 min after 1.43 mg/kg (Chart 2) and 120 min after 4.0 mg/kg, respectively (Chart 3). When evaluating the subsequent course of 7-methylguanine, it has to be taken into account that, 6.5 hr after the HU infusion, DNA synthesis rapidly decreases (39). Since DMN injection had been performed at 4 hr after HU infusion, a diluting effect of any residual DMA synthesis on the 7-methylguanine values in S phase can be excluded. This is certainly not the case in the present experiments. DNA replication leads to a diminution of the molar fraction of the bases. As a possibility to test whether formation and/or elimination of a base adduct are different in G1 and S, G1/S ratios of the molar fraction of the respective bases can be calculated. Those ratios are given in Tables 1 and 2. It is evident that the ratios in both dosage groups are initially almost identical for 7-methylguanine and 3-methyladenine, with higher values for 3-methyladenine at 120 and 240 min. The G1/S ratios for O6-methylguanine are different from both other alkylated bases, suggesting that formation and/or elimination of O6-methylguanine during DNA synthesis follow different rules. Another approach to correct for dilution of a methylated base because of DNA replication is to express the base as a molar fraction of 7-methylguanine as a function of time after DMN exposure. This takes for granted that 7-methylguanine elimination does not change during the cell cycle. For 10T½ cells in vitro, it has been reported recently that 7-methylguanine elimination ceases during DNA synthesis (53). However, in liver, no changes of 7-methylguanine glycosylase activity were observed in control and partially hepatectomized rats (36). Therefore, it seems justified to compare the results of G1 and S liver on the basis of O6-methylguanine/7-methylguanine and 3-methyladenine/7-methylguanine ratios.

### Table 2

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Interval (min)</th>
<th>7-Methylguanine</th>
<th>O6-Methylguanine</th>
<th>3-Methyladenine</th>
<th>14C-Guanine</th>
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<td>0.73</td>
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<td>0.62</td>
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<td>11.07</td>
<td>1.23</td>
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<td>1.83</td>
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</table>

*Between DMN injection and sacrifice of rats.

**Means of 1 to 3 experiments, each with 3 rats; molar fraction \( \times 10^4 \).**

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O6-Methylguanine elimination differences between G, and S compartments. In the G, group, the initial value of about $9 \times 10^{-2}$ 10 min after DMN (1.43 mg/kg) rises slightly, to about $11 \times 10^{-2}$. In contrast, the ratio drops rapidly in the synchronized S-phase compartment to reach a final value of about $5 \times 10^{-2}$ after 240 min (Chart 4). With the higher dose of 4 mg/kg, the ratio remains for G, cells at a slightly higher plateau. Cells in DNA synthesis show approximately the same ratio after 10 min for both dosages; but instead of a further decrease of the ratio as observed with the dosage of 1.43 mg/kg, a rise to about $9 \times 10^{-2}$ is observed at later intervals (Chart 5).

3-Methyladenine elimination differences between G, and S appear less apparent than for O6-methylguanine. In a plot of the 3-methyladenine/7-methylguanine ratio, scattering of data at early intervals precludes a correct conclusion. At later intervals, the ratio is lower for S-phase cells than for G, liver, indicating an enhanced elimination of 3-methyladenine during DNA synthesis (Chart 6).

Taking the G,/S ratios of the alkylated purines and the plots of O6-methylguanine/7-methylguanine and 3-methyladenine/...
7-methylguanine ratios for both dosages together, it can be concluded that both O6-methylguanine and 3-methyladenine are eliminated more rapidly in S phase, but the kinetics of these processes differs considerably. During DNA synthesis, initial elimination of O6-methylguanine proceeds much more rapidly than does the initial removal of 3-methyladenine as calculated on the molar basis (Tables 1 and 2) as well as seen in Charts 4 to 6. This rapid release is most pronounced with the lower DMN dose (Chart 4). After about 30 min, the rapid component of O6-methylguanine removal appears exhausted, and the rate of O6-methylguanine elimination becomes similar in G1 and in S. In contrast, 3-methyladenine elimination does not seem to be an exhaustable process; it proceeds at an approximately
equal rate during the whole period of observation but is faster during DNA synthesis.

**DISCUSSION**

It is particularly the liver which converts a large variety of carcinogen moieties (28) with an increased probability to interact directly at the site of origin with informational macromolecules of hepatocytes. Adducts at critical positions of DNA bases, quantitatively the most important being O6-methylguanine (31, 48), appear to be promutagenic (22). In the intact adult rat liver, the extraordinarily high DNA repair potential (32, 35) seems to be sufficient to remove most of these promutagenic lesions before they may lead to permanent changes of genetic information.

A critical exception is carcinogen exposure during DNA synthesis. During this phase, a single carcinogen dose induces tumors (8, 56). The increased risk of transformation during DNA synthesis in liver cells may be analogous to the situation in bacterial mutagenesis, where it has been shown that mutations arise preferentially at growing point regions (6). Alkylation at hydrogen-bonding sites of DNA bases induces base mispairing (1, 10, 27). There is not much objection against the assumption that the persistence in DNA of promutagenic lesions, as for instance O6-methylguanine, during DNA synthesis enhances the risk of GC → AT transition mutations (18) and thus could form the basis for permanent genetic changes and neoplastic transformation also in liver cells.

The results suggest that hepatocytes possess an increased potential for eliminating O6-methylguanine during DNA synthesis, and thus they appear to be able to reduce, although only to a limited extent, the levels of a potentially transforming lesion during this critical cell cycle phase. The mechanism of this O6-alkylguanine removal is still a matter of discussion. Since O6-methylguanine is stable in isolated DNA in vitro (20), the rapid elimination under the present conditions suggest an enzyme-catalyzed reaction. The different elimination kinetics of 3-methyladenine and O6-methylguanine indicates specific enzymes (11, 36, 49). A 3-methyladenine DNA glycosylase has been described which is different from the O6-methylguanine-eliminating principle (3, 44). The recorded half-life of about 3 hr for 3-methyladenine removal is in the range of the data of the literature (25, 26).

In a recent report, Pegg et al. (36) describe an increase of liver extract activity catalyzing *in vitro* the loss of O6-methylguanine 24 to 96 hr after partial hepatectomy with a maximum at 48 hr. The results presented in this paper suggest that such an O6-methylguanine-releasing activity is present *in vivo* in DNA-synthesizing liver cells, but not to the same extent in G1 cells. Inducibility (45, 46) and saturation seem to be peculiar facets of the O6-methylguanine-eliminating process. In mammalian cells, it is not yet fully understood whether the active principle is a DNA glycosylase or a de- or transalkylase (15, 21, 33). In mammalian cells, the capacity for O6-methylguanine removal appears to be saturated at rather low carcinogen concentrations [e.g. less than 0.5 μg/ml for N-methyl-N'-nitro-N-nitosoguanidine with human lymphoblastic cell lines (52)]. The rapid drop within 30 min of the O6-methylguanine/7-methylguanine ratio during S phase from the expected value of 0.11 (34) to a value of 0.05 is followed by a phase of slower O6-methylguanine elimination. A similar plateau-like phase, but at a higher level, is also observed after 4 mg/kg at later intervals after DMN. This rapid component of O6-methylguanine elimination is missing in G1 cells. This pattern suggests that the active principle which eliminates O6-methylguanine is present at a higher concentration (or activity) during S phase but is, in contrast to the 3-methyladenine DNA glycosylase, rapidly used up during the elimination process and thus could be equal to the adaptive O6-methylguanine elimination process described for the liver (29) and, in more detail, in bacteria (45, 46), where fast and slow elimination reactions were differentiated. The same seems to be the case in DNA-synthesizing liver cells. 3-Methyladenine, which is removed more rapidly from liver DNA during S phase (40) and for which an increased DNA glycosylase activity has been observed after partial hepatectomy (11), does not appear to be removed by a similar biphasic process like O6-methylguanine elimination.

A cell cycle dependence of base excision as well as of nucleotide excision repair has also been reported for WI-38 cells *in vitro*. The repair pathways were sequentially induced in the cell cycle prior to the induction of DNA synthesis (13, 51). Lack of 7-methylguanine and O6-methylguanine elimination during DNA synthesis has been claimed for C3H10T½ cells (53). In liver cells, the control of these repair processes may be different from cells *in vitro* (36). In bacteria, it has been shown that elimination of O6-methylguanine, but not of 3-methyladenine, requires protein synthesis (45, 55). The results obtained with regenerating rat liver indicate that synthesis of the material required for O6-methylguanine removal is concomitant with DNA synthesis (36). An increased repair has been observed at growing-point regions of DNA (47). However, the increased rate of O6-methylguanine elimination is apparently independent of the function of the DNA replication machinery itself. Studies in our group showed that in synchronized liver cells *in vivo* a DMN exposure during G2 + M results in a low O6-methylguanine/7-methylguanine ratio as compared with G1-exposed cells (40). This suggests that the active principle for a rapid O6-methylguanine removal from DNA, apparently synthesized during DNA synthesis, is still available during subsequent cell cycle phases. The same conclusion is suggested from Pegg’s data (36) about the O6-methylguanine eliminating activity of liver extracts after partial hepatectomy. Enhanced activity persisted at time periods after partial hepatectomy where DNA synthesis is already much diminished after a peak at 24 hr (36).

An induction by carcinogen pretreatment of an increased cellular capacity for O6-methylguanine elimination in the liver has been described (29, 36), even for nonrelated distinct classes of carcinogens (4) as well as an exhaustability of this potential (17). It is interesting to speculate about the possibility that this carcinogen-induced increment of O6-methylguanine repair capacity could be correlated with the well-known induction of DNA synthesis in the liver after carcinogen treatment (7, 38, 43). This would mean for the liver that the inductive step for the O6-methylguanine-eliminating enzyme is not a carcinogen-specific process but rather is dependent on induction of DNA synthesis, which, in turn, seems concomitant with the synthesis of the factor responsible for O6-methylguanine removal.

These considerations may be relevant also for the interpretation of a prolonged persistence of O6-alkylguanine in target cells after carcinogen exposure, as shown in particular for
brain cells exposed in vivo transplacentally prior to the birth of the embryos (12, 16). Reduction of DNA-synthetic activity in these target cells during the prenatal period might be coincident with a lack of inducibility of the rapid O6-alkylguanine elimination mechanism.

The intact liver appears to be partly protected against malignant transformation because of its high potential to remove O6-alkylguanine. Cell proliferation is certainly not an absolute requirement for repair to occur. However, it is an appealing thought that during DNA synthesis an even larger amount of an O6-methylguanine-eliminating enzyme could be made available for a replicating cell, in order to endow the cell with an additional means for fast removal of promutagenic DNA lesions and thus to reduce the probability of base mispairing during this critical cell cycle period. Rapid exhaustability of this repair process may explain why liver cells are transformed in spite of an increased O6-methylguanine repair during DNA synthesis.

However, conclusions with respect to malignant transformation as derived from the pattern of O6-alkylguanine formation and persistence alone may be premature. Other alkylation products, O2- and O4-thymine or O2-cytosine appear to be likewise promutagenic (48). There are indications that these alkylation products are removed enzymatically in replicating human cell lines in vitro (2), but data on the rate of repair during specific cell cycle phases are not yet available.

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