Effect of Chronic Treatment of Rats with Dimethylnitrosamine on the Removal of O\textsuperscript{6}-Methylguanine from DNA\textsuperscript{1}

Ruggero Montesano,\textsuperscript{2} Henriette Brésil, Ghyslaine Planche-Martel, Geoffrey P. Margison,\textsuperscript{3} and Anthony E. Pegg


ABSTRACT

Rats were treated chronically for up to 6 weeks with unlabeled dimethylnitrosamine at daily doses of 0.2 to 2 mg/kg/day. The effect of this pretreatment on the persistence of O\textsuperscript{6}-methylguanaine, 7-methylguanaine, and 3-methyladenine in DNA from liver, lung, and kidney was determined after administration of a single 2-mg/kg dose of [\textsuperscript{14}C]dimethylnitrosamine. Pretreatment did not affect the formation or the rate of loss of 3-methyladenine or 7-methylguanine in any organ but resulted in a lower initial amount of O\textsuperscript{6}-methylguanine in hepatic DNA as measured at 1 or 2 hr. The decrease in the initial amount of O\textsuperscript{6}-methylguanine was dependent on the dose of dimethylnitrosamine used for pretreatment, with 2 mg/kg/day giving a maximal effect, although a significant decrease could still be observed when 0.2 mg/kg/day was used. It was also related to the time of pretreatment, requiring at least 2 weeks of exposure to 2 mg/kg/day for a maximal response. There was no effect of O\textsuperscript{6}-methylguanine removal from DNA of kidney or lung at any dose used. Pretreatment with dimethylnitrosamine led to an increased activity of an enzyme removing O\textsuperscript{6}-methylguanine from methylated DNA in vitro which could be detected in liver extracts but had no effect on a similar activity in kidney extracts. These results suggest that the lower amount of O\textsuperscript{6}-methylguanine found in hepatic DNA of rats pretreated with dimethylnitrosamine was due to an increased activity of the enzyme system responsible for this reaction. This increase could be the result of an induction or activation of the enzyme in response to the chronic administration of the carcinogen. The results are discussed in relation to the carcinogenicity of dimethylnitrosamine for rat liver and to the observation of an inducible enzyme system in Escherichia coli, which carries out an analogous reaction and protects against mutagenesis by alkylating agents.

INTRODUCTION

A number of chemical carcinogens, including N-nitroso compounds, are known to act via their conversion by chemical decomposition or metabolic activation in the body into alkylating agents (22, 27), which react with various sites in DNA bases (30, 43, 44). Of these reaction products, O\textsuperscript{6}-alkylguanine is produced in greater yield or persists for longer periods of time in the DNA of the principal organs in which tumors arise than in the DNA of organs in which tumors do not occur. The active removal of O\textsuperscript{6}-methylguanine from DNA in vivo appears to be due to an enzymic mechanism (32, 33), which varies in activity from tissue to tissue and is lowest in the organs which are most susceptible to tumor induction (6, 9, 11, 14, 15, 19, 20, 23, 24, 29, 40). Furthermore, it has been suggested that the carcinogenic activity of alkylating agents is positively related to the amount of O\textsuperscript{6}-methylguanine produced (as a proportion of the total reaction products). The degree of formation of this alkylated base has been attributed to the mechanism in which the different alkylating agents react with DNA (17, 18).

Since most studies of this type have used single doses of carcinogens, it was considered to be worthwhile examining the activity of the enzymatic removal of O\textsuperscript{6}-alkylguanine excision mechanism during chronic treatment with carcinogens. The experimental model using DMN\textsuperscript{4} and the liver of rats was chosen because of the number of biochemical and carcinogenicity studies already available on this subject. These studies seemed to be of particular importance because most of the carcinogenicity tests are carried out using chronic administration of the carcinogen and exposure of humans to carcinogenic substances mainly occur chronically. Our previous studies (28, 34) suggested that an increase in the activity of this O\textsuperscript{6}-methylguanine excision system in the liver occurred after prolonged exposure to DMN. In the present experiments, this observation has been confirmed and extended by investigation of the dose and time dependence of the induction of the increase, by its organ specificity, and by measurements of the enzymatic activity in vitro with tissue extracts derived from the treated rats. The significance of such induction in carcinogenesis is also discussed.

MATERIALS AND METHODS

Materials. [\textsuperscript{14}C]DMN (5.18, 5.92, 7.4, or 16.25 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, United Kingdom, or from Paterson Laboratories, Manchester, United Kingdom, and unlabeled DMN was from Merck, Munich.

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\textsuperscript{2} To whom requests for reprints should be addressed.

\textsuperscript{3} Present address: Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, United Kingdom.

\textsuperscript{4} The abbreviation used is: DMN, dimethylnitrosamine.

\textsuperscript{5} The abbreviation used is: DMN, dimethylnitrosamine.

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Federal Republic of Germany. Sephadex G-10 was obtained from Pharmacia, Uppsala, Sweden. Other biochemicals were products of the Sigma Chemical Co., St. Louis, Mo., except for O6-methylguanine, which was synthesized by a published procedure (2). N\(^{14}\)H\(\text{Methyl-N-nitrosourea}\) (1.44 mCi/\(\mu\)mol) was purchased from NEN Corporation, Boston, Mass. \(\text{\textsuperscript{3}}\)H-Methylated DNA was prepared by reacting calf thymus DNA with \(40 \mu\text{M N\(^{14}\)H\text{methyl-N-nitrosourea}}\) for 30 min at pH 7.5. The resulting alkylated DNA was precipitated by addition of 2 volumes of 2-ethoxyethanol, washed 6 times with ethanol, dissolved in 10 mM Tris-HCl (pH 7.5), and dialyzed overnight. It was then used as a substrate for the enzyme assays.

**Experimental Design.** The experiments with BD IV rats were carried out at the International Agency for Research on Cancer, Lyon, France, and those with Sprague-Dawley rats were performed at the Department of Physiology and Specialization Cancer Research Center, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pa. Three groups of male BD IV rats (approximately 90 to 120 g at the start of the experiment) were used. Two groups were given unlabeled DMN by stomach tube (2.0 and 0.2 mg/kg, respectively) on weekdays (Monday to Friday) between 9 and 10 a.m. for a total of 44 days. The total dose administered was 88 or 8.8 mg/kg. Twenty-four hr after the last administration of unlabeled DMN, a 2-mg/kg dose of \([\text{\textsuperscript{14}}\text{C}]\text{DMN (5.18 mCi/mmol}}\) for the rats pretreated with 2 mg/kg/day and 7.4 mCi/mmol for those pretreated with 0.2 mg/kg/day) was given by stomach tube; the same dose of \([\text{\textsuperscript{14}}\text{C}]\text{DMN}\) was administered to a third control group of BD IV rats which had not received the DMN pretreatment. Two rats from each group were killed in pairs at various times, as specified, after the treatment with \([\text{\textsuperscript{14}}\text{C}]\text{DMN}\). The results from the rats receiving the pretreatment with 2.0-mg/kg doses DMN are reported in more detail elsewhere (28).

In another experiment, a group of female Sprague-Dawley rats weighing approximately 200 g received i.p. injections of unlabeled DMN (0.75 mg/kg) every day for a period of 5 weeks. \([\text{\textsuperscript{14}}\text{C}]\text{DMN (2 mg/kg)}\) was given 24 hr after the last injection, and the same dose of \([\text{\textsuperscript{14}}\text{C}]\text{DMN}\) was administered to another group of control rats not previously treated with unlabeled DMN. The rats were killed in pairs at various times after treatment with \([\text{\textsuperscript{14}}\text{C}]\text{DMN}\).

To examine the effect of the length of pretreatment, groups of 3 male BD IV rats (approximately 90 to 140 g at the start of the experiment) received a daily 2-mg/kg dose of unlabeled DMN by stomach tube during 1, 2, 3, 4, or 6 weeks (Monday to Friday). Twenty-four hr after the last administration of unlabeled DMN, \([\text{\textsuperscript{14}}\text{C}]\text{DMN (2 mg/kg; 16.25 mCi/mmol})\) was given i.p. The same dose of \([\text{\textsuperscript{14}}\text{C}]\text{DMN}\) was administered to a group of control rats not pretreated with unlabeled DMN. One control was carried out at the beginning of the experimental period, and one was performed at the end (6 weeks) to examine possible variations within this period. The rats were killed in pairs 6 and 24 hr after the treatment with labeled DMN. The livers, lungs, and kidneys were removed, frozen in liquid N\(_2\), and stored at \(-30^\circ\). One rat from each group was killed 24 hr after the last treatment with cold DMN; the liver was frozen in N\(_2\) and stored at \(-30^\circ\). This liver sample was used for the assay of enzymic activity removing O6-methylguanine from DNA.

In a separate experiment, 3 male BD IV rats received unlabeled DMN (2 mg/kg) by stomach tube for 3 weeks (Monday to Friday) and 24 hr afterwards were treated with \([\text{\textsuperscript{14}}\text{C}]\text{DMN (2 mg/kg; 5.92 mCi/mmol}}\); the rats were killed 10 min later. Two control rats received the \([\text{\textsuperscript{14}}\text{C}]\text{DMN only. The livers were removed, frozen in liquid N\(_2\), and stored at \(-30^\circ\).}

In other experiments to measure the enzyme activity in vitro, female Sprague-Dawley rats were used. They were treated with unlabeled DMN (0.75, 1.0, or 2.0 mg/kg i.p. daily) for 4 to 6 weeks as indicated. Extracts from liver and kidney were then prepared as described below and used for assays for enzymic activity.

**Preparation and Assay of Enzymic Activity Removing O6-Methylguanine from DNA.** Tissue extracts capable of catalyzing the removal of O6-methylguanine from alkylated DNA were prepared as described previously (32, 33). Assays were carried out by incubation of a total volume of 6 ml containing 0.5 mm (as phosphate) DNA substrate (20 \(\mu\text{mol of O6-methylguanine per mol guanine}\), 66 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, and 15 mg protein. After incubation at 37° for 1 hr, the DNA was precipitated by addition of 2 ml of cold 1 n perchloric acid and analyzed as described (32, 33). Control incubations in which no protein was added or reaction was halted at zero time showed that no significant loss of O6-methylguanine occurred in the absence of protein. The recovery of guanine and 7methylguanine in the acid-precipitable DNA after incubation with the tissue extracts was more than 95% of that added, showing that the loss of O6-methylguanine from the acid-precipitable DNA was due to a specific process and not the action of a nonspecific nuclease.

**Isolation and Analysis of DNA.** DNA was isolated by extraction with phenol as previously described (25) and stored at \(-30^\circ\). The DNA was hydrolyzed to release free purine bases by heating at 70° for 30 min in 0.1 n HCl. After addition of unlabeled markers of methylated purines (3-methyladenine, 7methylguanine, and O6-methylguanine), the sample was adjusted to pH 2.9 and then chromatographed on a column of Sephadex G-10 (85 x 1.0 cm) eluted with 0.05 M ammonium formate, pH 6.75. The radioactivity present in the fractions corresponding to methylated bases was determined. The amounts of guanine and adenine in the sample analyzed were calculated from the absorbance of the fraction containing these bases. The amounts of methylated bases present were then calculated as \(\mu\text{mol/mol of the parent base}\). Further details of this analytical procedure are given in recent publications (25, 28, 31, 35).

**RESULTS**

**Effects of the Dose of Pretreatment.** Table 1 shows the amounts of various alkylated purines (7-methylguanine, 3methyladenine, and O6-methylguanine) in liver DNA various times after administration of \([\text{\textsuperscript{14}}\text{C}]\text{DMN (2 mg/kg)}\) to control rats or to rats which had received unlabeled DMN at a dose of 0.2 mg/kg/day for 44 days or at a dose of 0.75 mg/kg/day for 35 days. Pretreatment with DMN did not affect the levels of 7-methylguanine or 3-methyladenine observed at the earliest time point measured or at subsequent time points. The loss of 7-methylguanine from DNA was very slow during the 12-hr period, whereas 3-methyladenine had decreased by 75% over the same time span. This was observed in both pretreated and control animals. These results indicate that the pretreatment did not alter either the ability of the liver to metabolize the

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**DMN Induction of DNA Repair**

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nitrosamine to form an alkylating intermediate or the rate of loss of these products from DNA.

Less O\(^6\)-methylguanine was observed at all times after \(\text{'C} \text{DMN}\) treatment in the liver DNA of rats receiving the DMN pretreatment as compared to control rats. The differences in the relative amounts and persistence of this alklylation product are shown more clearly in the O\(^6\)-methylguanine/7-methylguanine ratios (Chart 1). These ratios, based on the radioactivity present in different fractions from the same column eluate, are probably more accurate than the individual measurement of alklylated bases, since they do not depend on the determination of the actual amount of the parent base present in DNA. As shown in Chart 1, the O\(^6\)-methylguanine/7-methylguanine ratio in the control groups at 2 hr was about 0.085, and it decreased to about 0.070 by 12 hr. In the liver DNA of pretreated rats (0.2 mg/kg/day), the initial (1-hr) ratio was 0.074 and decreased to 0.044 by 12 hr. When the pretreatment dose was 0.75 mg/kg/day, the ratio at 2 hr was 0.053; by 12 hr, it had fallen to 0.032. A still greater reduction in the initial amount of O\(^6\)-methylguanine was observed when rats were pretreated with higher doses of DMN (2 mg/kg/day) (Chart 1; Ref. 28). As indicated in "Materials and Methods," these results were obtained in 2 different strains of rat (BD IV and Sprague-Dawley), suggesting the general nature of the phenomenon.

No change in the rate of loss of 3-methyladenine was observed in any of the pretreated rats, as indicated by the similarity between the 3-methyladenine/7-methylguanine ratios of the liver DNA from control and treated rats at the different dose levels of DMN pretreatment (Chart 2).

The activity of cell-free preparations from liver and kidney which were able to remove O\(^6\)-methylguanine from methylated DNA in vitro was determined using extract from control and DMN-treated rats (Table 2). A significant increase in activity was produced by pretreatment with DMN in liver, but not in kidney. The activity in liver was enhanced 2- to 3-fold by pretreatment with 1 to 2 mg/kg/day for 4 weeks or 0.75 mg/kg/day for 6 weeks.

Effect of the Length of Pretreatment. The amounts of 7-methylguanine, 3-methyladenine, and O\(^6\)-methylguanine in liver DNA 6 hr after administration of \(\text{'C} \text{DMN}\) (2 mg/kg) to control rats or to rats which had received unlabeled DMN (2 mg/kg) during 1, 2, 3, 4, or 6 weeks are shown in Table 3. No difference was observed in the levels of 7-methylguanine and 3-methyladenine between control and pretreated rats. Therefore, pretreatment did not affect alkylation at the 7-position of guanine or in the 3-position of adenine. The similar levels of O\(^6\)-methylguanine in the 2 control groups which were killed at 1 and 6 weeks after the start of the experiment indicate that no variation occurred within the experimental period that may have influenced the formation and persistence of this methylated base in the DNA. On the other hand, the treatment with DMN resulted in a lower level of labeled O\(^6\)-methylguanine within 1 week of treatment. A still lower amount of O\(^6\)-methylguanine was found by 2 weeks of pretreatment, and no further significant decline was observed by continuing the pretreatment up to 6 weeks (Table 3).

The amounts of the various alkylated purines present 24 hr

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**Table 1**

Alkylated purines in rat liver DNA at various times following administration of \(\text{'C} \text{DMN}\)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>7-Methylguanine</th>
<th>3-Methyladenine</th>
<th>O(^6)-Methylguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-treated Control</td>
<td>Pre-treated Control</td>
<td>Pre-treated Control</td>
<td></td>
</tr>
<tr>
<td>A*</td>
<td>1</td>
<td>633</td>
<td>632</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>660</td>
<td>612</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>658</td>
<td>638</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>625</td>
<td>607</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>603</td>
<td>599</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>584</td>
<td>571</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>562</td>
<td>575</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>496</td>
<td>508</td>
</tr>
</tbody>
</table>

* The data at 6 and 12 hr in the control rats are taken from the paper of Montesano et al. (28).
Table 2
Effect of pretreatment with various doses of DMN on activity of enzyme removing O\textsuperscript{6}-methylguanine from DNA

Female Sprague-Dawley rats weighing 150 g at the start of the experiment were treated with the doses of DMN shown for 4 weeks (1 and 2 mg/kg) or 6 weeks (0.75 mg/kg), and the tissue extract was prepared as described under "Materials and Methods." Results for liver samples are shown as mean ± S.D. for 4 or 5 samples. Results for kidney are mean of 2 estimations each run in duplicate.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Activity (pmol/100 mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Liver</td>
</tr>
<tr>
<td>None</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>DMN (0.75 mg/kg)</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>DMN (1.0 mg/kg)</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>DMN (2.0 mg/kg)</td>
<td>24 ± 3</td>
</tr>
</tbody>
</table>

Table 3
Alkylated purines in liver DNA of BD IV rats 6 hr after administration of \[^{14}C\]DMN

Alkylated purines were measured in liver DNA of BD IV rats pretreated during different periods with unlabeled DMN (2 mg/kg) and measured 6 hr after administration of \[^{14}C\]DMN (2 mg/kg; 16.25 mCi/mmol). Controls received \[^{14}C\]DMN only.

<table>
<thead>
<tr>
<th>Time of pretreatment (wk)</th>
<th>7-Methylguanine</th>
<th>3-Methyladenine</th>
<th>O\textsuperscript{6}-Methylguanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreated</td>
<td>Control</td>
<td>Pretreated</td>
<td>Control</td>
</tr>
<tr>
<td>1</td>
<td>428</td>
<td>441</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>370</td>
<td>411</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>450</td>
<td>450</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>523</td>
<td>523</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>502</td>
<td>504</td>
<td>16</td>
</tr>
</tbody>
</table>

DMN Induction of DNA Repair

Chart 3. Alkylation in liver DNA of BD IV rats after pretreatment for 1 or several weeks (5 days/week) with unlabeled DMN (2 mg/kg) and administration of \[^{14}C\]DMN at a dose of 2 mg/kg. Control rats received \[^{14}C\]DMN only. 7-Methylguanine (7-meG) (A, A) and O\textsuperscript{6}-methylguanine (O\textsuperscript{6}-meG) (O, O, O, control rats; O, pretreated rats). O\textsuperscript{6}-methylguanine/7-methylguanine (O, O, O, O, control rats; O, pretreated rats). G, guanine. The values of the control rats are the mean of the controls carried out at the beginning and at the end (6 weeks) of the experimental period.

Table 4
Alkylated purines in rat liver DNA 10 min after administration of \[^{14}C\]DMN

Alkylated purines were measured in liver DNA of BD IV rats pretreated for 5 days/week with unlabeled DMN (2 mg/kg) and 1 administration of \[^{14}C\]DMN at a dose of 2 mg/kg. Control rats received \[^{14}C\]DMN only.

<table>
<thead>
<tr>
<th>Methylated bases (μmol/mol of parent base)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Methylguanine</td>
</tr>
<tr>
<td>Pretreated</td>
</tr>
<tr>
<td>154</td>
</tr>
</tbody>
</table>

In the above experiments, it is clear that the maximum effect is present after 3 weeks of pretreatment, and under these conditions a lower amount of O\textsuperscript{6}-methylguanine in liver DNA as compared to controls was detected as early as 10 min after the treatment with \[^{14}C\]DMN (2 mg/kg) (Table 4).

The effect of length of pretreatment with DMN (2 mg/kg/day) on the enzymic activity as measured in cell extracts was also studied (Chart 4). Pretreatment for 1 week produced a 50% increase in the enzyme activity removing O\textsuperscript{6}-methylguanine from alkylated DNA in vitro. After 2 weeks, the activity was increased by almost 100%, and there was only a slight further increase with an apparent maximum about 2.2 times that in the control rat, which did not significantly change during the course of the experiment. It should be noted that the activities from both control and maximally induced rats in Chart 4 were slightly less than those in Table 2. This is probably because the activities in Chart 4 were measured with extracts from liver frozen immediately after death and stored at −70° for some time, whereas those in Table 2 were measured on extracts isolated immediately from fresh tissue. However, strain and sex differences cannot be ruled out because the former experiment used male BD IV rats and the latter used female Sprague-Dawley rats.
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(2 mg/kg) for 6 weeks did not affect the formation of \( \text{O}^6 \)-methylguanine from DNA in vivo. This result is in agreement with the data shown in Table 2. The activity of the extracts in catalyzing the removal of \( \text{O}^6 \)-methylguanine from the DNA in control rats and from rats pretreated for up to 6 weeks with DMN (2 mg/kg/day) (•) as in Table 3. The activity of the extracts in catalyzing the removal of \( \text{O}^6 \)-methylguanine from alkylated DNA was then determined as described in Table 2.

The levels of 7-methylguanine and \( \text{O}^6 \)-methylguanine present in DNA 6 hr after injection of \(^{14} \text{C}\text{DMN}\) were also measured in kidney and lung DNA. Pretreatment with daily doses of DMN (2 mg/kg) for 6 weeks did not affect the formation of 7-methylguanine in these tissues, showing that metabolic conversion to the alkylation species was not altered (data not shown). Furthermore, as shown by the similar \( \text{O}^6 \)-methylguanine/7-methylguanine ratios in the control rats and rats pretreated with the nitrosamine (Table 5), there was no significant effect on the excision of \( \text{O}^6 \)-methylguanine from the DNA in these organs. This result is in agreement with the data shown in Table 2, in which the ability of kidney extracts to remove \( \text{O}^6 \)-methylguanine from DNA \textit{in vitro} was measured and found not to be influenced by pretreatment with DMN.

**DISCUSSION**

The presence of \( \text{O}^6 \)-alkylguanine in DNA during DNA replication may be an important factor in the initiation of tumors by DMN and related alkylating carcinogens (6, 9, 11, 14, 15, 19, 20, 23, 24, 29, 40). Therefore, the rapidity of removal of this product from DNA may be an important protective mechanism against carcinogenesis, provided that a substantial degree of removal can occur prior to DNA synthesis. The experimental evidence regarding the removal from DNA of \( \text{O}^6 \)-methylguanine indicates that: (a) this product is chemically stable \textit{in vitro} under physiological conditions but is lost from DNA \textit{in vivo} at a rate which is higher than explicable by DNA turnover (26, 30, 38); (b) there are different rates of loss from the DNA of various organs of the same animal species (6, 9, 11, 14, 15, 19, 20, 23, 24, 29, 40) or in the same organ of different animal strains (3, 4, 7) and species (24, 45); and (c) certain tissue extracts are able to catalyze specifically the loss of \( \text{O}^6 \)-methylguanine, but not 7-methylguanine, from DNA \textit{in vitro} (33—35). These observations suggest that an enzymic process is responsible for the removal of \( \text{O}^6 \)-alkylguanine from DNA \textit{in vivo}.

The findings reported here show that continuous exposure to low doses of DMN results in a lower amount of \( \text{O}^6 \)-methylguanine in liver DNA of exposed rats than in liver DNA of rats not thus exposed. Although the precise rates of removal of \( \text{O}^6 \)-methylguanine from DNA have not been measured in this series of experiments, previous results (28) have shown that not only was the initial (2-hr) level of \( \text{O}^6 \)-methylguanine much lower than in control animals but also the subsequent rate of loss between 2 and 12 hr was more rapid than in the control animals. Since there is no reason to suppose that the number of molecules of \( \text{O}^6 \)-methylguanine actually generated in DNA is altered by pretreatment, a lower amount found in DNA of pretreated animals must indicate an increased rate of removal compared with controls at some early time between administration and measurement of the amounts of \( \text{O}^6 \)-methylguanine.

The \( \text{O}^6 \)-methylguanine/7-methylguanine ratio (which gives an indication of the relative amount of \( \text{O}^6 \)-methylguanine removed) determined 12 hr after a single dose of \(^{14} \text{C}\text{DMN}\) (2 mg/kg) varied from about 0.074 in control rats to 0.024 in rats pretreated with 2 mg/kg/day. Pretreatment with lower doses of DMN had an intermediate effect. The values in the pretreated animals may have been influenced by the repair of DNA containing \( \text{O}^6 \)-methylguanine produced by the preceding doses of unlabeled DMN and therefore give a conservative indication of the measurement of the magnitude of the effect, especially in the case of the higher pretreatment dose. However, even if this is so, the results show a dose-response relationship. No differences were observed in the rate of loss of 3-methyladenine or 7-methylguanine from DNA in control and pretreated animals, indicating that the effect is specific for \( \text{O}^6 \)-methylguanine. Variation in the length of pretreatment indicated that the enhanced activity was produced within 2 weeks of pretreatment with DMN.

Within this time period, no significant change in the cell population within the liver is likely to be present, as a consequence of toxicity, even after the highest dose of pretreatment (2 mg/kg), and it certainly is not present after the 0.2-mg/kg dose of DMN given for 1 week. If variation of cell population was responsible for the observed difference, an even greater effect and one affecting equally the 3 alklylation products should have been observed with the longer period of pretreatment (see Chart 3). Certainly, this factor should be taken into account, when other dose levels or a longer schedule of administration of DMN are used.

The reduced amount of \( \text{O}^6 \)-methylguanine present in liver DNA following chronic pretreatment with low doses of DMN

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**Table 5**

\( \text{O}^6 \)-Methylguanine/7-methylguanine ratios in kidney and lung DNA of BD IV rats 6 hr after administration of \(^{14} \text{C}\text{DMN}\)

<table>
<thead>
<tr>
<th>Time of pretreatment (wk)</th>
<th>Kidney</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.058</td>
<td>0.057</td>
</tr>
<tr>
<td>2</td>
<td>0.082</td>
<td>0.054</td>
</tr>
<tr>
<td>3</td>
<td>0.043</td>
<td>0.072</td>
</tr>
<tr>
<td>4</td>
<td>0.051</td>
<td>0.092</td>
</tr>
<tr>
<td>6</td>
<td>0.065</td>
<td>0.036</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.050</td>
<td>0.092</td>
<td>0.067</td>
</tr>
</tbody>
</table>
might be the result of the induction of the enzyme(s) involved. A similar effect may occur in rats receiving multiple doses of 3,3-dimethyl-1-phenyltriazene (4). Investigation of the removal of O6-methylguanine from rat liver DNA after a wide range of single doses of DMN indicated that there may be more than one enzyme system for this process, one of which is saturated at much lower levels of DNA damage than the other (36). The present results show that at least one such enzyme system which can be measured in an in vitro assay system is increased by pretreatment with DMN in vivo. This result is consistent with an induction of the liver enzyme, but the increased activity was only 2- to 3-fold greater than in the control liver, and an activation mechanism must also be considered. Recent studies on the mechanisms of the enzymic removal of O6-methylguanine from DNA suggest that it may involve a methyl transfer to an acceptor molecule, which is regenerated sufficiently slowly that its availability becomes the rate-limiting factor. Increased activity could therefore result from an increase in the level of this acceptor. Whatever the mechanism of the increase, the present results are in marked contrast to the inhibition of O6-methylguanine excision produced by large single doses of alkylating agents (16, 29, 31, 35).

Increased activity of the O6-methylguanine systems was observed to follow pretreatment with DMN only in liver and not in kidney or lung. However, it should be remembered that the degree of alkylation occurring in these tissues following administration of the carcinogen is much less than in the liver (22, 29, 30), particularly after p.o. administration of DMN at low doses (8, 34). It is therefore possible that a greater interaction of the carcinogen with kidney and lung than was provided in the present studies might result in an increased activity.

The increased excision of O6-methylguanine from liver DNA of rats treated with low doses of nitrosamine may be related to the recent observation in Escherichia coli of an inducible error-free repair process during exposure to low doses of the alkylating agent, N-methyl-N'-nitro-N-nitrosoguanidine. E. coli exposed to sublethal concentrations of alkylating agents, like N-methyl-N'-nitro-N-nitrosoguanidine, develop a resistance to their mutagenic effects (13, 41). This resistance was shown to be due to an inducible error-free repair process, distinct from the SOS repair process (37, 48) and specifically affecting certain DNA alkylation products. It could not be induced by other DNA-damaging agents, like UV irradiation or 4-nitroquinoline 1-oxide. Recently, it was found (39, 42) that the kinetics of mutagenesis in bacteria pretreated with N-methyl-N'-nitro- N-nitrosoguanidine parallels the appearance of O6-methylguanine in DNA, these cells having increased ability to remove this DNA adduct from their DNA in comparison with normal cells. No such parallelism was observed for other DNA bases, such as 7-methylguanine and 3-methyladenine. The enzymic repair system for O6-methylguanine in E. coli also resembles the system found in rat liver in that both are saturated or function much less efficiently when too high a degree of alkylation is achieved (16, 31, 42). However, the parallels between the 2 systems should not be overemphasized since there are important differences in both the much slower increase in the hepatic activity which requires 2 weeks for a near maximal effect and in the magnitude of the increase which is only 2- to 3-fold in liver, but much more than this in the bacteria.

Long-term carcinogenicity studies6 with a large range of dose levels of DMN indicate that, above a certain dose level of DMN, the liver cancer risk in rats increases rapidly with the dose of DMN administered, resulting in at least a 1000-fold increase of tumors with a 10-fold increase in daily dose rate. The analysis of a preceding study on the carcinogenicity of DMN in rat liver (46) is in keeping with these observations (12). This type of carcinogenic response is consistent with enzymic repair of O6-methylguanine in liver DNA during continuous treatment with various dose levels of DMN. Liver cells have one or more enzyme systems which function specifically to repair O6-methylguanine lesions in DNA (31, 33–35). These observations show that this enzyme(s) may be increased in activity by low-level exposure to alkylating agents but that high doses of these agents may exceed the capacity of even the induced enzymes to repair DNA, thus substantially increasing the risk of cancer in animals receiving large doses. Further studies are in progress to elucidate this correlation quantitatively. It is important to stress that the presence of an enzyme system removing O6-methylguanine from DNA more efficiently after lower doses of the carcinogen (28, 31) does not imply the existence of a threshold dose below which no significant carcinogenic action of DMN would be expected. As discussed recently by Weinhouse (47), there is no convincing biochemical evidence for the existence of true threshold doses in chemical carcinogenesis. The results reported here merely suggest that, at some point, risk of cancer developing would increase more rapidly than in proportion to the dose rate of the carcinogen.

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Effect of Chronic Treatment of Rats with DimethylNitrosamine on the Removal of $O^6$-Methylguanine from DNA

Ruggero Montesano, Henriette Brésil, Ghyslaine Planche-Martel, et al.


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