Accumulation of *O*⁶-Methylguanine in Non-Target-Tissue Deoxyribonucleic Acid during Chronic Administration of Dimethylnitrosamine

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1. BD-IV rats were given labelled dimethylnitrosamine (2mg/kg) by stomach tube on weekdays (Monday to Friday) for up to 24 weeks. The rats were killed after 2, 4, 8, 16 and 24 weeks of treatment (72h after the final dimethylnitrosamine gavage) and DNA was isolated from the pooled livers, kidneys and lungs. Purine bases were released from the DNA by mild acid hydrolysis and separated by Sephadex G-10 chromatography. 2. Throughout the experiment, the content of 7-methylguanine in liver DNA was approx. 16 times that in kidney and lung. The amount of this product increased in the DNA of all three tissues up to 16 weeks, but by 24 weeks had decreased by 20% in the liver and 46% in the other tissues. 3. *O*⁶-Methylguanine was not detected in liver DNA, but was easily measured in kidney and lung DNA after 4 weeks of dimethylnitrosamine administration. The amount of *O*⁶-methylguanine in kidney and lung DNA increased relative to that of 7-methylguanine, and by 24 weeks was 60% of the 7-methylguanine content in both tissues. 4. Incorporation of radioactive C₁ breakdown products of dimethylnitrosamine into normal purines in DNA increased continuously in all three tissues. 5. The results are discussed with respect to the specific hepatocarcinogenic effect of chronic administration of dimethylnitrosamine and the possible contribution of increased DNA repair and DNA synthesis.

The organ specificity of the carcinogenic effect of dimethylnitrosamine in the rat changes according to the dose schedule; kidney tumours are induced if dimethylnitrosamine is given in a high toxic dose either singly or over a short period, whereas liver is the target tissue if dimethylnitrosamine is given repeatedly in a low dose for a protracted period. This change in organ specificity was clearly demonstrated by Magee & Barnes (1962), who administered dimethylnitrosamine in the diet at several different concentrations. There are also some indications of strain differences in the organ specificity of chronically administered dimethylnitrosamine; in Sprague-Dawley and Wistar rats, lung and kidney tumours were reported in some studies but not in others (Zak *et al.*, 1960; Argus & Hoch-Ligeti, 1961; Hadjiolov, 1968; Magee *et al.*, 1976). However, in BD rats, only liver tumours have been found after chronic dimethylnitrosamine administration (Druckrey *et al.*, 1967).

Dimethylnitrosamine is converted *in vivo* into an alkylating agent after metabolism by microsomal mixed-function oxidases. This process, which in rats

occurs principally in the liver and to a lesser extent in kidney and lung, results in the alkylation of cellular macromolecules, including DNA, RNA and protein (Magee *et al.*, 1975). Alkylation occurs at several positions in DNA, but one of the minor products, *O*⁶-methylguanine, has received particular attention, since the suggestion that it might miscode (Loveless, 1969) has been supported by the results of experiments *in vitro* using synthetic polyribo- or polydeoxyribo-nucleotide templates (Gerchman & Ludlum, 1973; P. Abbott & R. Saffhill, personal communication).

There is now some evidence that the organ specificity of these agents is related to the capacity of the tissues to remove this product enzymically from DNA (see Kirtikar & Goldthwait, 1974), the lowest activity being that of the target organs (Goth & Rajewsky, 1974; Kleihues & Margison, 1974; Nicoll *et al.*, 1975; Margison & Kleihues, 1975; Margison *et al.*, 1976b). Thus after a single dose of dimethylnitrosamine rat liver was shown to be able to remove *O*⁶-methylguanine from DNA (O'Connor *et al.*, 1973). Similarly, this base was lost rapidly from both liver and kidney DNA after a dose of 2.5mg of dimethylnitrosamine/kg, whereas after a dose of 20mg/kg, which produces a high incidence of kidney tumours, the capacity of the kidney to

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excise *O*⁶-methylguanine was greatly decreased (Nicoll *et al.*, 1975). Although the repair capacity of liver for *O*⁶-methylguanine excision is the highest on those rat tissues so far studied, it was found to be less efficient after split doses of alkylating agents (Kleihues & Margison, 1976). It has been found that weekly application of methylnitrosourea to rats at a dose that induces a very high incidence of tumours of the nervous system resulted in an accumulation of *O*⁶-methylguanine in brain DNA (Margison & Kleihues, 1975). In the present experiments we have determined the amounts of *O*⁶-methylguanine in the DNA of target and non-target tissues of BD rats during chronic dimethylnitrosamine administration to discover to what extent repeated doses might similarly result in accumulation of this product.

Materials and Methods

Chemicals

[¹⁴C]Dimethylnitrosamine (35 mCi/mmol), provided by Dr. J. A. Kepler, Research Triangle Institute, Research Triangle Park, NC, U.S.A., was diluted to a specific radioactivity of 0.492 mCi/mmol (0.418 mg/ml) by using unlabelled dimethylnitrosamine (Merck-Schuchardt, Munich, Germany). Sephadex G-10 was from Pharmacia, Uppsala, Sweden.

Animal experiments

Male BD-IV rats (approx. 90 g at the start of the experiment, 5 weeks old) were given [¹⁴C]dimethylnitrosamine (2 mg/kg body wt. per day) by stomach tube on weekdays (Monday to Friday) between 08:30 and 09:00 h. Rats were killed in pairs at 2, 4, 8, 16 and 24 weeks after the first dimethylnitrosamine gavage, i.e. 72 h after the final dimethylnitrosamine treatment in each case. The actual accumulated doses per rat were 2.5, 6.1, 15.6, 38.5 and 62.1 mg respectively.

DNA extraction

Organs were removed, frozen in liquid N₂ and stored at -30°C. DNA was isolated from pooled organs by a phenol procedure as follows. Tissues were homogenized in 6–10 vol. of a solution containing 6% (w/v) 4-aminosalicylate and 1% (w/v) NaCl, then shaken vigorously for 30 min with an equal volume of a mixture consisting of phenol (1 kg), *m*-cresol (150 ml), 8-hydroxyquinoline (1.0 g) and water (110 ml). After centrifugation at 5°C and 15000g for 30 min, the upper layer was removed and 3 mg of NaCl added/ml. The mixture was re-extracted with 0.5 vol. of the phenol mixture and centrifuged as described above. To the upper layer was added 2 vol. of cold ethanol/*m*-cresol (9:1, v/v), and after 30 min the precipitate was removed, washed three times with

at least 20 ml of cold 70% (v/v) ethanol containing 2% (w/v) sodium acetate, then redissolved in water. This solution was made 3 M in NaCl and left at 4°C overnight. The precipitated RNA was removed by centrifugation at 12000g for 20 min and washed with 3 × 10 ml of 3 M-sodium acetate. To the pooled supernatants was added 2 vol. of 2-ethoxyethanol and the precipitated DNA was again washed with ethanol/sodium acetate and redissolved in water. Bovine pancreatic ribonuclease (0.4 ml of a 2 mg/ml solution) and saturated sodium acetate (0.15 ml) were added and the mixture was incubated overnight at 4°C. Glycogen was removed by centrifugation at 60000g for 60 min at 4°C and the purified DNA precipitated with ethoxyethanol, washed with ethanol/sodium acetate (three times), ethanol (twice), ethanol/diethyl ether (1:1, v/v) and ether, then dried in a stream of N₂ and stored at -30°C.

DNA analysis

Purine bases were released from DNA by hydrolysis in 0.1 M-HCl (approx. 1 ml/5 mg of DNA) at 70°C for 30 min (Lawley & Thatcher, 1970). After addition of authentic 7-methylguanine and *O*⁶-methylguanine, the sample was adjusted to pH 2.9 by using 0.1 M-NaOH, then applied to a column (85 cm × 1 cm) of Sephadex G-10 eluted at 15 ml/h with 0.05 M-NH₃/0.2% (w/v) NaN₃ adjusted to pH 6.75 with formic acid. Fractions (4.5 ml) were collected and the *A*₂₆₀ measured for the determination of normal purines (absorption coefficient 7.2 litre · mol⁻¹ · cm⁻¹ for guanine, 13.3 litre · mol⁻¹ · cm⁻¹ for adenine). Radioactivity was determined in the purine-containing fractions after evaporation in a stream of air, dissolution of the residue in 0.3 ml of Hyamine (1 M solution in methanol) and the addition of 10 ml of toluene containing 0.5% (w/v) 2,5-diphenyloxazole. Counting efficiency was determined by quench correction and was about 83%. Results for normal and methylated purines were expressed as d.p.m./μmol of the parent base. Repeat determinations differed by less than 6%.

Results

The amounts of 7-methylguanine and *O*⁶-methylguanine in the DNA of liver, lung and kidney of rat during chronic administration of [¹⁴C]dimethylnitrosamine are shown in Table 1. Even though there was a decrease in the amount of 7-methylguanine in DNA between weeks 16 and 24 of treatment, the relative amounts of this product in lung and kidney DNA were 6.06 ± 1.58% and 6.36 ± 1.05% of the values in liver respectively, showing no consistent changes. The higher extent of methylation of liver DNA compared with lung and kidney reflects the higher capacity of the liver for metabolism of dimethylnitrosamine.

Table 1. Amounts of 7-methylguanine and O⁶-methylguanine (d.p.m./μmol of guanine) in rat tissue DNA during chronic administration of [¹⁴C]dimethylnitrosamine (0.492 mCi/mmol; 2 mg/kg per day on weekdays). Animals were killed 72 h after the final dimethylnitrosamine application. DNA was extracted from the pooled tissues and analysed as described in the Materials and Methods section.

Time (weeks)	Radioactivity (d.p.m./μmol of guanine)					
	7-Methylguanine			O ⁶ -Methylguanine		
	Liver	Lung	Kidney	Liver	Lung	Kidney
2	512	38	33	0	0	0
4	679	42	40	0	5.0	8
8	737	53	47	0	5.0	11
16	923	55	74	0	13	22
24	764	26	39	0	15	24

Fig. 1. O⁶-/N-7-methylguanine ratio in rat tissue DNA during chronic oral administration of [¹⁴C]dimethylnitrosamine (0.492 mCi/mmol; 2 mg/kg per day on weekdays). Details were as in Table 1. ●, Liver; ▲, kidney; ■, lung.

O⁶-Methylguanine was present in lung and kidney DNA in measurable amounts after 4 weeks of dimethylnitrosamine administration, and it continued to increase in amount throughout the experiment. However, no O⁶-methylguanine was detected in the DNA of liver even after 24 weeks treatment (Table 1).

The increase in the O⁶-/N-7-methylguanine ratio in the DNA of lung and kidney during the course of dimethylnitrosamine administration (Fig. 1) indicates the specific accumulation of this base in these tissues. The large difference in the three tissues is clearly demonstrated in Fig. 2, which shows the Sephadex G-10 elution profiles of the acid-hydrolysed DNA from the liver, lung and kidney of rats treated for 24 weeks with dimethylnitrosamine. Closely similar amounts of

Fig. 2. Purine-containing regions from Sephadex G-10 elution profiles of 0.1 M-HCl hydrolysates of DNA from the tissues of rats treated with [¹⁴C]dimethylnitrosamine (0.492 mCi/mmol; 2 mg/kg per day on weekdays) for 24 weeks (a) Liver; (b) kidney; (c) lung. In (a) the radioactivity scale has been adjusted to give the same peak height for 7-methylguanine in all three profiles. Inset: the O⁶-methylguanine region on the same d.p.m. scale as in (b) and (c). Peaks: I, 7-methylguanine; II, guanine; III, adenine; IV, O⁶-methylguanine.

DNA were analysed, and the d.p.m. scales have been adjusted so that the peak heights for 7-methylguanine are the same. The relative (and absolute; see inset, Fig. 2) amounts of O⁶-methylguanine in

Fig. 3. Radioactivity in adenine (d.p.m./ μ mol of adenine) in rat tissue DNA during chronic oral administration of [14 C]dimethylnitrosamine (0.492 mCi/mmol; 2 mg/kg per day on weekdays)

Details were as in Table 1. ●, Liver; ▲, kidney; ■, lung.

liver DNA are negligible compared with those of lung and kidney.

The incorporation of radioactivity into the normal purines in DNA increased consistently and almost in parallel in all three tissues throughout the experiment (see Fig. 3). However, that in kidney was somewhat lower than that of lung or liver.

Discussion

The metabolism of dimethylnitrosamine *in vivo* leads to the production of an alkylating intermediate, probably the methylcarbonium ion, which attacks various positions in DNA. The major site of reaction is the 7-position of guanine but the 3- and O^6 -positions of guanine, the 1-, 3- and 7-positions of adenine, the 3- and O^4 -positions of thymine and 3- and O^2 -positions of cytosine as well as the phosphodiester groups are all susceptible to attack (Lawley, 1974; Singer, 1976). The relative initial amounts of these products are determined by the reactivity of the alkylating agent and do not vary from tissue to tissue. However, O^6 -methylguanine in the system so far investigated has been shown to persist or accumulate in the tissues most susceptible to tumour induction (Goth & Rajewsky, 1974; Kleihues & Margison, 1974; Nicoll *et al.*, 1975; Margison & Kleihues, 1975; Margison *et al.*, 1976b).

Of the rat tissues so far studied, the liver has the

highest capacity for O^6 -methylguanine removal, but the rate of loss of this base was decreased when large amounts were produced in liver DNA (Kleihues & Margison, 1974). Recent reports agree with these findings (Pegg, 1977). Split doses of alkylating agents were also very effective in decreasing the capacity of the liver for O^6 -methylguanine excision (Kleihues & Margison, 1976). However, it was shown that chronic administration of much lower doses of dimethylnitrosamine had no effect on the capacity of the liver for O^6 -methylguanine removal (Margison *et al.*, 1976a).

During weekly administration of methylnitrosourea to rats, a dose schedule that induces a very high incidence of tumours in the central nervous system, some kidney tumours, but no liver tumours (Druckrey *et al.*, 1967), O^6 -methylguanine accumulated to the greatest extent in the DNA of brain, to a lesser extent in kidney, and none was more detected in liver DNA (Margison & Kleihues, 1975). In the present experiments, we have investigated the possibility that the chronic administration of dimethylnitrosamine might similarly allow accumulation of this base in target-organ DNA. The amounts of O^6 -methylguanine in the DNA of liver, lung and kidney have thus been determined after up to 24 weeks of oral application of dimethylnitrosamine to BD rats, a strain in which only liver tumours have been induced by such treatment (Druckrey *et al.*, 1967).

The major product of alkylation of DNA by dimethylnitrosamine is 7-methylguanine. In contrast with O^6 -methylguanine, this product is lost from rat tissue DNA by a non-enzymic process (spontaneous fission of the *N*-glycosidic bond), which occurs in different tissues at the same rate (Kleihues & Margison, 1974; Nicoll *et al.*, 1975). The amounts of 7-methylguanine in DNA can therefore be used as a measure of the alkylation reaction and the ratio O^6 -/*N*-7-methylguanine as a measure of the relative O^6 -methylguanine-excision rates in different tissues, since differences in cell turnover etc. will affect both products equally (Margison & Kleihues, 1975; Nicoll *et al.*, 1975; Kleihues & Margison, 1976).

The amounts of 7-methylguanine in the DNA of liver, lung and kidney increased in the first 16 weeks of [14 C]dimethylnitrosamine administration, but by 24 weeks had decreased by 20% in the liver and 46% in the other two organs (Table 1). In the liver, this may have been due, at least partly, to an increase in the rate of DNA synthesis, which has been demonstrated by an increase in the incorporation of labelled adenine (Craddock, 1970) or C_1 breakdown products from labelled methylnitrosourea (G. P. Margison & R. Montesano, unpublished work) into liver DNA after chronic dimethylnitrosamine administration. This increased DNA synthesis is possibly a consequence of the toxic effects of dimethylnitrosamine (Terracini *et al.*, 1967), resulting in compensatory restorative

hyperplasia. Another possible cause of decreased DNA methylation could be the inhibition of the dimethylnitrosamine-metabolizing enzymes. This has been shown to occur in mouse liver during chronic dimethylnitrosamine treatment (Den Engelse & Emmelot, 1971/72), but to what extent it takes place in rat tissues has not yet been ascertained. It is noteworthy that, in the present experiment in all three tissues, metabolic labelling of purine shows no decrease between 16 and 24 weeks (Fig. 3).

Although large amounts of 7-methylguanine were present in liver DNA, no O⁶-methylguanine was detected throughout the period of the experiment (Table 1). Thus chronic administration of dimethylnitrosamine does not lead to a specific accumulation of O⁶-methylguanine in target-organ DNA. In fact, the contrary appears to be true, as O⁶-methylguanine was found to accumulate in the DNA of lung and kidney, which are not target organs in BD rats, after chronic dimethylnitrosamine administration. This is clearly seen in the O⁶-/N-7-methylguanine ratios, which increased continuously in lung and kidney DNA, the greatest increase being between 16 and 24 weeks, and suggesting a more marked accumulation of O⁶-methylguanine during this period (Fig. 1).

These results appear not to support the hypothesis that O⁶-methylguanine is important in the carcinogenic effect of alkylating agents; however, two factors must be considered. First, the experiment was designed to measure the possible accumulation of products in DNA and consequently, there was a 72 h interval between the final dose of dimethylnitrosamine and the animals' death. At 5 h after dimethylnitrosamine injection, the O⁶-/N-7-methylguanine ratio in DNA is 0.14 (Margison *et al.*, 1976b): from the relative extents of alkylation in the various tissues and the rate of loss of 7-methylguanine from DNA *in vivo* (Margison *et al.*, 1973), it can be calculated that in the present experiment 30–40 times more O⁶-methylguanine has been produced in (and excised from) liver DNA than from the DNA of the other tissues. The chronic production of this DNA damage will, compared with a single dose of dimethylnitrosamine, considerably increase the chance of alkylation taking place in a cell undergoing DNA synthesis. This could be analogous to the single-dose hepatocarcinogenicity of dimethylnitrosamine administered during premitotic DNA synthesis after partial hepatectomy (Craddock, 1971). Secondly, differential toxicity during chronic dimethylnitrosamine administration will considerably increase the number of sensitive ('target') cells compared with normal liver cells. The mechanism of this effect may be similar to that described by Farber *et al.* (1976) and Becker (1975) in rats, showing that hyperplastic nodules induced by some hepatocarcinogens are more resistant to the acute cytotoxicity of dimethylnitros-

amine or carbon tetrachloride than the surrounding non-nodular liver and a higher yield of tumours is obtained by the additional dimethylnitrosamine treatment.

An alternative, or additional, explanation for the present observations is that DNA undergoing extensive repair (i.e. in the liver) may be a more critical site of DNA damage. It could be envisaged that if, during excision repair (induced by one dose of dimethylnitrosamine), O⁶-methylguanine was produced (by a subsequent dose of dimethylnitrosamine) in the single-stranded region of DNA about to undergo repair replication, miscoding would be inevitable. Such an event might not arise in the tissues in which DNA alkylation and repair are not so extensive.

In conclusion, the present results suggest that, although O⁶-methylguanine may be the critical product of alkylating carcinogens, the induction of tumours in certain tissues does not always correlate simply with the persistence or accumulation of this product in DNA and that the effects of DNA synthesis or other alkylation products (such as O⁴-methylthymidine or phosphotriesters) should be taken into account in experiments of this kind.

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