Chemical Carcinogenesis in the Nervous System

PREFERENTIAL ACCUMULATION OF *O*⁶-METHYLGUANINE IN RAT BRAIN DEOXYRIBONUCLEIC ACID DURING REPETITIVE ADMINISTRATION OF *N*-METHYL-*N*-NITROSOUREA

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(Received 13 January 1975)

1. The alkylation of purine bases in DNA of several rat tissues was determined during weekly injections (10mg/kg) of *N*-[³H]methyl-*N*-nitrosourea, a dose schedule known to selectively induce tumours of the nervous system. Each group of animals was killed 1 week after the final injection, and the DNA hydrolysates were analysed by chromatography on Sephadex G-10. 2. After five weekly applications, *O*⁶-methylguanine had accumulated in brain DNA to an extent which greatly exceeded that in kidney, spleen and intestine. In the liver, the final *O*⁶-methylguanine concentration was less than 1% of that in brain. Between the first and the fifth injection, the *O*⁶-methylguanine/7-methylguanine ratio in cerebral DNA increased from 0.28 to 0.68. In addition, 3-methylguanine was found to accumulate in brain DNA whereas in the other organs no significant quantities of this base were detectable. 3. The results are compatible with the hypothesis that *O*⁶-alkylation of guanine in DNA plays a major role in the induction of tumours by *N*-methyl-*N*-nitrosourea and related carcinogens. 4. The kinetics of the increase of *O*⁶-methylguanine in cerebral DNA suggest that there is no major cell fraction in the brain which is capable of excising chemically methylated bases from DNA. This repair deficiency could be a determining factor in the selective induction of nervous-system tumours by *N*-methyl-*N*-nitrosourea and other neuro-oncogenic compounds.

N-Methyl-*N*-nitrosourea is a potent carcinogen which, under certain dose schedules, selectively induces tumours of the nervous system (Druckrey *et al.*, 1967). The carcinogenicity of *N*-methyl-*N*-nitrosourea has been attributed to its ability to alkylate nucleic acids in the intact animal. Among the various reaction products, *O*⁶-alkylguanine in DNA has received particular attention, since this base has been suggested to be promutagenic (Loveless, 1969) and was found to miscode in an RNA polymerase system *in vitro* (Gerchman & Ludlum, 1973). There is recent indication that the capacity of different rat tissues to remove *O*⁶-alkylguanine from DNA may correlate with the organ-specific carcinogenic effect of *N*-methyl-*N*-nitrosourea and related alkylnitrosoureas (Goth & Rajewsky, 1974; Kleihues & Margison, 1974). After a single injection of *N*-methyl-*N*-nitrosourea in adult rats, *O*⁶-methylguanine is eliminated more slowly from cerebral DNA than from that of kidney and liver (Kleihues & Margison, 1974). However, a single dose (60–80mg/kg) causes the development of tumours in various rat tissues, whereas the selective induction of nervous-

system tumours has been shown to follow repetitive low doses (Druckrey *et al.*, 1965, 1967; Jänisch & Schreiber, 1969; Swenberg *et al.*, 1972). We have therefore determined the concentrations of *O*⁶-methylguanine in DNA of various tissues during weekly injections of *N*-[³H]methyl-*N*-nitrosourea (10mg/kg), i.e. a dose schedule which eventually would have led to the production of nervous-system tumours in most experimental animals. Under these conditions, *O*⁶-methylguanine was found to accumulate in brain DNA to an extent which greatly exceeded that of any other organ investigated.

Experimental

Animals

Female BD-IX rats (Druckrey, 1971) were used. At the beginning of the experiment, body weights were 103–109 g.

Chemicals

N-[³H]Methyl-*N*-nitrosourea (45.5mCi/mmol) was obtained from NEN Chemicals, Dreieichenhain, West Germany. For injection it was dissolved in

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3 mM-sodium citrate (pH 6.0). Unlabelled *N*-methyl-*N*-nitrosourea was added to give a specific radioactivity of 26.3 mCi/mmol. Bovine pancreatic ribonuclease (EC 3.1.4.22) was obtained from Boehringer, Mannheim, West Germany. For use it was dissolved in water (2 mg/ml) and heated to 80°C for 10 min to inactivate contaminating deoxyribonucleases.

Animal experiments

N-[³H]Methyl-*N*-nitrosourea was injected into the femoral vein under light ether anaesthesia at a dose of 10 mg/kg. Animals were divided into five groups, each consisting of two rats. Group 1 received a single injection and was killed 1 week later. The other groups received two to five injections at weekly intervals and were killed 1 week after the final injection.

Isolation of DNA

Animals were killed by exsanguination under ether anaesthesia. Organs were frozen in liquid N₂ and stored at -70°C. DNA was isolated by phenol extraction as follows. Tissues were allowed to thaw in 6–10 vol. of 6% (w/v) sodium *p*-aminosalicylate containing 1% NaCl, and subsequently homogenized by hand in a loose-fitting Teflon-glass homogenizer. The homogenate was shaken with an equal volume of phenol reagent (phenol-*m*-cresol-8-hydroxyquinoline-water, 100:14:0.1:11, w/v/w/v) for 30 min at room temperature (22°C). After centrifugation for 30 min at 4°C and 16000g, the phenol phase was similarly re-extracted with 0.5 vol. of the sodium *p*-aminosalicylate-NaCl mixture. The combined supernatants were made 3% (w/v) with respect to NaCl and extracted with 0.5 vol. of the phenol reagent. To the viscous aqueous phase was added 2 vol. of ethanol-*m*-cresol (9:1, v/v), and after repetitive inversions the precipitate was spooled out on to a glass rod and extracted with 3 ml of 3.0 M-sodium acetate. After centrifugation for 20 min at 13000g and 5°C, the precipitate was extracted twice more with 3 ml of sodium acetate and the crude DNA precipitated from the combined supernatants by addition of 2 vol. of cold 2-ethoxyethanol. The DNA was dissolved in 3 ml of water and dialysed against 2 litres of water for approx. 4 h at 4°C. RNA contaminants were degraded by overnight incubation at 4°C after addition of ribonuclease solution (0.4 ml, 2 mg/ml) and saturated sodium acetate (0.15 ml). The purified DNA was precipitated with 2 vol. of 2-ethoxyethanol, washed several times with cold ethanol, dried and stored at -20°C.

Analysis of purine bases

DNA (5–10 mg) was hydrolysed in 1.0 ml of 0.1 M-HCl at 37°C for 17 h (Lawley & Thatcher,

1970). The purine bases were loaded on columns (90 cm × 1 cm) of Sephadex G-10 and eluted with 0.05 M-ammonium formate, pH 6.8 (Lawley & Shah, 1972). Authentic marker compounds (3-methyladenine, 3-methylguanine, 7-methylguanine and *O*⁶-methylguanine) were added to some hydrolysates. Their elution positions relative to adenine were identical with those reported by Lawley & Shah (1972), except for 3-methyladenine, the elution of which depends on the pH of the applied sample (Goth & Rajewsky, 1974). After the extinction at 260 and 280 nm had been measured, fractions (4.5 ml) were transferred to plastic counting vials and dried in a stream of air. The residue was dissolved in 0.3 ml of 1 M-Hyaminate hydroxide in methanol and counted for radioactivity for 50 min after the addition of 10 ml of toluene containing 0.5% (w/v) 2,5-diphenyloxazole and 0.05% (w/v) *p*-bis-(*o*-methylstyryl)benzene. Quench correction was by external standardization, counting efficiency being approx. 40%. The background radioactivity was stable at 29–31 d.p.m./fraction.

Amounts of methylguanines present were expressed as mol% of guanine, taking the molar extinction coefficient of guanine at neutral pH to be 7200 litre·mol⁻¹·cm⁻¹ and assuming that their specific radioactivity was the same as that of the methyl group of the injected *N*-[³H]methyl-*N*-nitrosourea (Swann & Magee, 1968; Lawley & Shah, 1973). The amount of radioactivity in the *O*⁶-methylguanine peak varied from 0 to 42 d.p.m. (liver) and 1200 to 4300 d.p.m. (brain). Occasionally, guanine and adenine were not completely separated. The *E*₂₈₀/*E*₂₆₀ ratios of these bases at neutral pH are 1.02 and 0.11 respectively. It was therefore possible to calculate the relative amounts of guanine and adenine in each of the overlapping fractions.

By using pairs of animals, the variation in the amount of 7-methylguanine determined in different experiments was found to be less than 6%. The estimation of minor alkylation products may vary to a greater extent according to the absolute amount of radioactivity present.

Results

Fig. 1 shows elution profiles from Sephadex G-10 of DNA hydrolysates from animals which received five weekly injections of *N*-[³H]methyl-*N*-nitrosourea. Marked differences can be seen in the pattern of alkylated purines in liver and brain. In both organs, 7-methylguanine is the major reaction product, but in the brain there are also substantial amounts of *O*⁶-methylguanine, measurable quantities of 3-methylguanine and traces of 3-methyladenine. These minor alkylation products are virtually absent from the liver. The absolute amounts of 7-methylguanine

Fig. 1. Fractionation of the purine bases of DNA from rat liver (a) and brain (b)

Animals received five weekly injections of *N*-[³H]methyl-*N*-nitrosourea (10mg/kg) and were killed 1 week after the final injection. DNA was isolated, hydrolysed and chromatographed on Sephadex G-10 as described in the text (fraction volume was 4.5ml). 3-MeAde, 3-Methyladenine; 3-MeGua, 3-methylguanine; 7-MeGua, 7-methylguanine; Gua, guanine; Ade, adenine; O⁶-MeGua, O⁶-methylguanine; O, radioactivity (d.p.m.); ●, E_{260} .

Table 1. Alkylated purines in DNA from different rat tissues after repeated administration of *N*-[³H]methyl-*N*-nitrosourea

Two BD-IX rats received five injections of *N*-[³H]methyl-*N*-nitrosourea (10mg/kg; 26.3mCi/mmol) at weekly intervals and were killed 1 week after the final dose. Isolation and analysis of DNA was as described in the text.

Organ	10 ⁴ × Mol % of guanine		Ratio O ⁶ -MeGua/ 7-MeGua
	O ⁶ -Methyl- guanine	7-Methyl- guanine	
Brain*	59.70	88.42	0.675
Kidney	13.93	61.35	0.227
Spleen	4.86	31.21	0.156
Small intestine	2.79	22.25	0.125
Liver	0.45	43.72	0.010

* In brain DNA, 3-methylguanine was also present (8.7×10^{-4} mol% of guanine).

and O⁶-methylguanine in the DNA from these and other tissues are given in Table 1.

The amounts of O⁶-methylguanine in brain, kidney and liver DNA during repeated application of

N-[³H]methyl-*N*-nitrosourea over a 5-week period are shown in Fig. 2. In the brain, O⁶-alkylation of guanine exhibits an approximately linear increase with time. In the kidney, O⁶-methylguanine amounts to about 25% of that in brain DNA, whereas in the liver no significant accumulation is apparent.

Fig. 3 shows the time-course of O⁶-alkylation when expressed as a fraction of (*N*)7-alkylation of guanine, complemented with data obtained in previous experiments (Kleihues & Margison, 1974). In the brain, the O⁶-methylguanine/7-methylguanine ratio increases steadily, whereas in kidney and liver this ratio shows a rapid initial decrease. In the kidney, this is followed by an increase, which tends to reach a plateau after 3–5 weeks.

Discussion

In the past, several attempts have been made to find a correlation between the tissue-specific carcinogenicity of *N*-nitroso compounds and their ability to alkylate nucleic acids of different organs *in vivo*. No consistent differences were found to exist in the initial extent of alkylation between target and non-target tissues (Swann & Magee, 1968, 1971; Goth & Rajewsky, 1972; Kleihues & Magee, 1973), but the

Fig. 2. O^6 -Methylguanine in DNA during repeated applications of N -[3H]methyl- N -nitrosourea

Animals received one to five injections (each of 10mg/kg) at weekly intervals and were killed 1 week after the final injection. The first dose was given at zero time. Brain (●); kidney (■); liver (▲).

conclusions reached in these studies were based mainly on the amounts of the major reaction product, 7-alkylguanine. It is now realized that some minor alkylation products, in particular O^6 -alkylguanine, may be more significant in the mutagenic and carcinogenic effect of this group of compounds (see review by Lawley, 1974). Although the relative initial extent of O^6 -alkylation of guanine could explain the differences in the carcinogenic potency of agents such as N -methyl- N -nitrosourea and methyl methanesulphonate (Kleihues & Magee, 1973; Lawley, 1974), it does not account for the selective production of tumours in certain organs. There are, however, indications that O^6 -alkylguanine, in contrast with 7-alkylguanine is removed from DNA by an enzymic repair process (O'Connor *et al.*, 1973; Craddock, 1973; Kirtikar & Goldthwait, 1974) and that the rate of excision may vary in different tissues according to their susceptibility to the carcinogenic effect of alkylating agents (Goth & Rajewsky, 1974; Kleihues & Margison, 1974).

This concept is strongly supported by the present experiment. During weekly applications of N -methyl- N -nitrosourea (10mg/kg), a dose schedule that leads to the selective production of nervous-system tumours (Druckrey *et al.*, 1965), O^6 -methylguanine accumulated in brain DNA to a much greater extent than in kidney, spleen or intestine (Fig. 2 and Table 1). These latter organs do occasionally develop tumours

Fig. 3. Changes in the O^6 -methylguanine/7-methylguanine ratio in DNA during repeated applications of N -[3H]methyl- N -nitrosourea

The dose schedule was as in Fig. 2. Brain (●, ○); kidney (●, □); liver (▲, △). The 6h and 78h values (open symbols) are taken from Kleihues & Margison (1974). The asterisk at zero time represents the hypothetical initial O^6 -methylguanine/7-methylguanine ratio as found after reaction of N -methyl- N -nitrosourea with DNA *in vitro* (Lawley, 1974).

after a single high dose (60–80mg/kg) of N -methyl- N -nitrosourea (Druckrey *et al.*, 1967). In the liver, which is not susceptible to the carcinogenicity of N -methyl- N -nitrosourea (except after partial hepatectomy; Craddock & Frei, 1974), the amount of O^6 -methylguanine at the end of the experimental period was less than 1% of that in brain DNA.

These results can only partially be explained by differences in the rates of cell turnover, since the latter would proportionally decrease the amount of 7-methylguanine: when O^6 -alkylation of guanine is expressed as a fraction of (N)7-alkylation, the differences between the tissues become somewhat smaller, the O^6 -methylguanine/7-methylguanine ratio in cerebral DNA being between three times (kidney) and 60 times (liver) as high as in other organs (Table 1). To a greater extent, the tissue differences seem to reflect the capacity of the various organs actively to remove O^6 -alkylation damage. If N -methyl- N -nitrosourea is caused to react with DNA in the absence

of cellular repair processes *in vitro*, the O⁶-methylguanine/7-methylguanine ratio is 0.1 (Lawley, 1974). Fig. 3 shows that in liver and kidney this ratio is probably never achieved *in vivo* owing to a very rapid initial excision of O⁶-methylguanine. The inability of the brain to repair the chemical modification of its DNA may not be restricted to O⁶-alkylation of guanine, since distinct quantities of 3-methylguanine were also found to have accumulated in cerebral DNA (Fig. 1). Alkylation in the (N)3-position of guanine has been observed after reaction of N-methyl-N-nitrosourea with DNA *in vitro* (Lawley *et al.*, 1972) and in liver DNA after treatment with dimethyl-nitrosamine *in vivo* (Craddock, 1973).

The presence of small amounts of 3-methyladenine in brain DNA 1 week after the last injection (Fig. 1) is difficult to explain since this product is released from DNA by non-enzymic hydrolysis, a reaction which proceeds with a half-life of 24 h at 37°C in aqueous solutions *in vitro* (Lawley & Brooks, 1963). The amount of radioactive N-methyl-N-nitrosourea injected should not allow the detection of this base after 7 days if a similar half-life applied to the situation *in vivo*.

After a single dose of N-methyl-N-nitrosourea (10 mg/kg), O⁶-methylguanine in cerebral DNA decreased by approx. 20% within 3 days (Kleihues & Margison, 1974). The present study indicates that this rate of loss is not maintained as part of an exponential decay curve and may therefore reflect the activity of only a minor cell population. The approximately linear accumulation of O⁶-methylguanine in cerebral DNA during a 5-week period of repetitive application strongly suggests that a significant excision of this base does not occur in any major cell fraction of the brain.

The technical assistance of Mrs. U. Winkler and Miss I. Stachelhaus is gratefully acknowledged. G. P. M. was a recipient of a Max-Planck Fellowship.

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