Carcinogenesis vol.19 no.5 pp.731–739, 1998

DNA adducts, mutant frequencies and mutation spectra in λ*lac*Z transgenic mice treated with N-nitrosodimethylamine

Vassilis L.Souliotis[1], Joost H.M.van Delft[2], Marie-José S.T.Steenwinkel[2], Robert A.Baan[2] and Soterios A.Kyrtopoulos[1,3]

[1]Laboratory of Chemical Carcinogenesis, Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, 48 Vassileos Constantinou Ave., Athens 11635, Greece and [2]TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ Zeist, Utrechtseweg 48, 3704 HE Zeist, The Netherlands

[3]To whom correspondence should be addressed
Email: skyrt@eie.gr

Groups of λ*lac*Z transgenic mice were treated i.p. with N-nitrosodimethylamine (NDMA) as single doses of 5 mg/kg or 10 mg/kg or as 10 daily doses of 1 mg/kg and changes in DNA *N*7- or *O*^{6}-methylguanine or the repair enzyme *O*^{6}-alkylguanine-DNA alkyltransferase (AGT) were followed for up to 14 days in various tissues. Adduct induction in the liver exceeded by at least one order of magnitude than observed in the next nearest target tissue (lung), and was approximately linearly related to dose, except for *O*^{6}-methylguanine after the first dose of 1 mg/kg which was lower than expected. Substantial induction of λ*lac*Z mutagenesis was observed only in the liver, where the mutant frequency was already maximal within 7 days after 5 mg/kg NDMA and remained unchanged thereafter up to 49 days. Small but marginally significant increases in mutant frequency were consistently observed in the spleen after all three modes of treatment. A lack of proportionality between mutation induction and the administered dose or the corresponding adduct levels was observed, probably reflecting the importance of toxicity-related cell proliferation caused by NDMA at higher doses. Twenty eight days after a dose of 10 mg/kg (causing a 3.6-fold increase in mutant frequency), NDMA was found to increase the frequency of GC→AT mutations (with a concomitant shift of their preferential location from CpG sites to GpG sites), which made up ~60% of the induced mutations. Surprisingly, NDMA also caused a significant increase in deletions of a few (up to 11) base-pairs (22%).

Introduction

N-Nitrosodimethylamine (NDMA*) is a carcinogen of substantial laboratory and environmental interest, capable of inducing benign and malignant tumours in many animal species and in a variety of tissues, including the liver, the kidney, the lung and the nasal cavity (1,2). Human exposure to NDMA is widespread, associated primarily with nitrate- or nitrite-treated foods, certain beverages and tobacco smoke (3,4). Significant human exposure is also derived from endogenous formation of NDMA in the stomach and possibly in other body compartments (5–8). Accurate, direct estimation of endogenous exposure to NDMA is not possible, but, based on indirect evidence, it has been suggested that this mode of exposure may greatly exceed that arising from exogenous sources and may constitute a significant cancer risk factor for man (9,10). Although epidemiological studies have provided evidence compatible with a carcinogenic role of NDMA in man, such evidence is far from conclusive, possibly because the effectiveness of these studies was compromised by the difficulty of estimating the true extent of total NDMA exposure (11–18). Hence, and in view of the powerful and multispecies animal carcinogenicity of NDMA, there is a need to add mechanism-based approaches to epidemiological investigations of the role of NDMA in human cancer.

NDMA has been widely employed as a model experimental carcinogen and for this reason much is known about the mechanism of its biological effects. It is metabolised primarily in the liver by cytochrome P4502E1 to a methylating reactive intermediate (probably the methyldiazonium ion) which methylates cellular macromolecules (19). It is an S_N1-type methylating agent, generating in DNA primarily *N*7-methylguanine (*N*7-meG, ~70% of all adducts formed), *O*^{6}-methylguanine (*O*^{6}-meG, ~7%), *N*3-methyladenine (~3%), *O*^{4}-methylthymine (*O*^{4}-meT <0.1%), methylphosphotriesters (~12%) as well as a number of other minor base modifications (20). The quantitatively major DNA lesion *N*7-meG is not directly mutagenic, but it can undergo enzymatic or spontaneous depurination to form mutagenic apurinic sites (21). On the other hand, oxygen-centred base methylation adducts are directly mutagenic and most current evidence suggests that the most abundant of these, *O*^{6}-meG, plays a major role in mutagenesis and carcinogenesis by NDMA and other S_N1-type methylating agents. This is supported by correlations between the accumulation of *O*^{6}-meG in different animal tissues or cell types and susceptibility to methylating-agent carcinogenesis, as well as by the cancer protective effect of the repair enzyme *O*^{6}-alkylguanine-DNA alkyltransferase (AGT) which specifically repairs *O*^{6}-meG (22–25).

Single-adduct studies have demonstrated that *O*^{6}-meG is strongly premutagenic, giving rise to GC→AT transitions by direct miscoding during cell replication (26–28), a sequence change which has been found to dominate the mutation spectra induced *in vitro* and *in vivo* by S_N1 methylating agents (29–31). In the case of NDMA, sequence analysis of large numbers of mutants derived from mutagenesis in the *lac*I gene of *Escherichia coli* has shown that it induces predominantly GC→AT transitions, as well as a small proportion (~10%) of other types of mutations (30,32).

While information on the mutagenicity of NDMA in whole animals is relatively limited, recent studies with *lac*Z or *lac*I transgenic mice have shown that it is mutagenic mainly in the liver and, to a smaller degree, the lung and kidney, but not in the bone marrow, urinary bladder or testis (33–35). Furthermore, it has recently been reported that administration of NDMA in

***Abbreviations:** NDMA, N-nitrosodimethylamine; *O*^{6}-meG, *O*^{6}-methylguanine; AGT, *O*^{6}-alkylguanine-DNA alkyltransferase; *O*^{4}-meT, *O*^{4}-methylthymine; *N*7-meG, *N*7-methylguanine; P-gal, phenyl-β-galactoside; WBC, white blood cells; MF, mutation frequency.

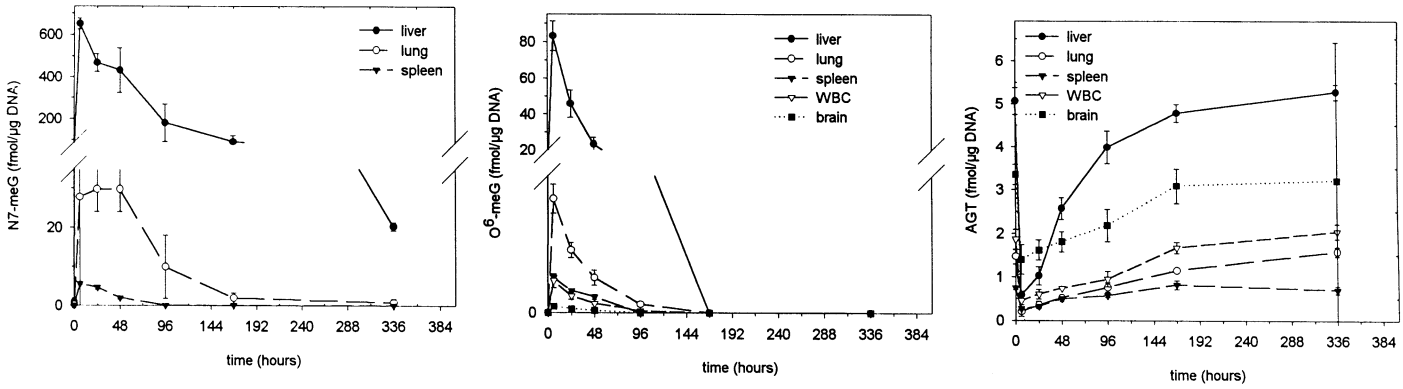

Fig. 1. Changes in *N*7- and O^6-meG and in AGT in various tissues of mice following a single dose of 5 mg/kg NDMA. Data are averages obtained from 4 mice. For experimental details, see Materials and methods.

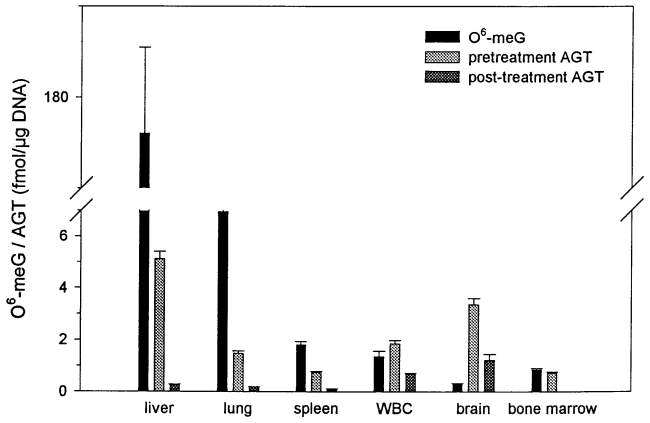

Fig. 2. Levels of O^6-meG and AGT 6 h following a single dose of 10 mg/kg NDMA, as well as pre-treatment levels of AGT, in various tissues of mice. The levels of *N*7-meG in the liver after the same treatment were 1591 ± 247 (SD). Data are averages obtained from 4 mice. For experimental details, see Materials and mthods.

the form of split doses or even chronic treatment in the drinking water is more effective in inducing liver mutations than administration of the same amount as a single dose (33,36). However, as no DNA adduct data were reported in these studies, interpretation of dose effects and extrapolation to events likely to occur under environmentally relevant conditions is difficult. As regards the types of mutations induced by NDMA *in vivo*, limited information comes from Mirsalis *et al.* (35) who reported that eight out of 10 liver mutations induced in *lac*I transgenic mice by five daily doses of 4 mg/kg NDMA were GC→AT transitions.

GC→AT mutations have also been found to occur frequently in cancer-related genes of animal tumours induced by NDMA and other methylating N-nitroso compounds (37–43), thus implying an important role for O^6-meG in carcinogenesis by such agents. On the other hand, it is notable that in a number of cases human cancers for which, on the basis of epidemiological studies, the etiological involvement of N-nitroso compounds (including NDMA) is suspected, the mutations in the corresponding genes are not dominated by GC→AT transitions (44–47). Although these observations may indicate lack of involvement of NDMA in tumour induction, they also invoke the possibility that under certain conditions NDMA may induce a more complex pattern of mutations than that observed under the conditions employed in experimental studies.

In the work reported here an attempt was made to begin addressing the question of the mechanism of NDMA mutagenesis *in vivo*. For this purpose, λ*lac*Z transgenic mice were exposed to single or multiple doses of NDMA and the kinetics of induction of DNA methylation adducts and mutations in different tissues, as well as the spectrum of induced mutations, examined. We report that, following single or multiple treatment of animals with NDMA with doses ranging up to 10 mg/kg, (i) a clear mutagenic effect was observed only in the liver, while a small effect was consistently observed in the spleen, (ii) as the dose was increased, liver mutagenesis increased more than adduct accumulation and (iii) after a dose of 10 mg/kg NDMA, in addition to the predominating GC→AT mutations a significant proportion of single- and multiple-base deletions were also induced in the liver.

Materials and methods

Animal experiments

Male Muta^{TM} mice, 9–10 weeks old (Hazleton) were maintained on normal laboratory chow. For each dose or time point, groups of four animals were treated i.p. with NDMA dissolved in physiological saline. Animals were killed at appropriate times after treatment and tissues immediately placed in liquid nitrogen. For AGT measurement in white blood cells, the cells were isolated from fresh blood by lysis of the erythrocytes in 155 mM NH_4Cl/10 mM $KHCO_3$/0.1 mM EDTA on ice for 15 min, followed by centrifugation and washing of the leukocyte pellet with PBS, prior to freezing. All tissues were stored at –70°C until analysis.

Measurement of O^6-meG, N7-meG and AGT

DNA was isolated by a previously reported method involving multiple treatments with proteinase K and RNase and phenol/chloroform extraction (48). Measurement of O^6-meG was carried out by the competitive repair assay (49), with a limit of detection, using 10 µg DNA per assay, of 0.05 fmol/µg DNA (0.08 µmol/molG). *N*7-meG was analysed by hydrolysis of DNA (enzymatic or by heat treatment) prior to HPLC with electrochemical detection or by immunoslot-blot methodology using monoclonal antibodies, with a limit of detection of 0.6 fmol/µg DNA (1 µmol/molG) (50). AGT was determined in tissue extracts using as substrate ^{3}H-methylated calf thymus DNA (48).

Mutant frequency analysis

λ Phages containing the *lac*Z gene were rescued from high molecular weight DNA using a commercially available phage packaging extract (Giga-pack II Gold, Stratagene) and subsequently used to infect *E.coli lac*ZgalE bacteria (51). Bacteria containing *lac*Z-mutated phages were selected using phenyl-β-galactoside (P-gal). In most cases >400 000 phages were analysed, while groups of four animals were used for each time-point.

Sequencing of mutants

To determine the region (α, β, ω) of the β-galactosidase gene in which the mutation was located, a β-galactosidase protein complementation assay was carried out involving replating of the mutants on the three complementation strains of *E.coli*: DH5a (α–, β+, ω+), W4680 (α+, β–, ω+) and Hfr3000×90 (α+, β+, ω–) (52). The region thus determined to contain the mutations was

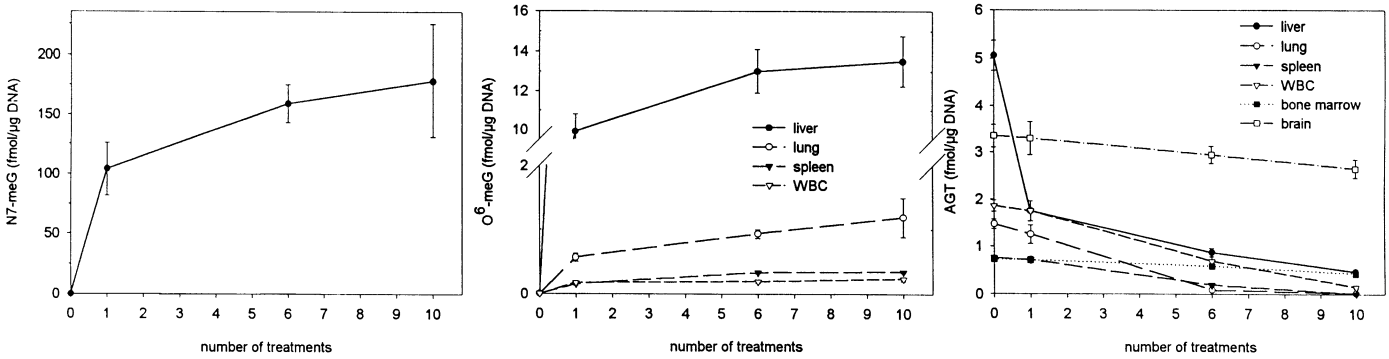

Fig. 3. Changes in *N*7-meG (only in the liver), O^6-meG and AGT (various tissues) during 10 daily treatments of mice with 1 mg/kg NDMA. Data are averages obtained from 4 mice. For experimental details, see Materials and methods.

subjected to PCR amplification using pairs of primers of which one was 5′-biotinylated. Following separation of PCR products by low melting agarose gel electrophoresis, binding of the biotinylated PCR products to magnetic beads and DNA sequence analysis was performed by standard methods (53).

Results

In order to assess the dose- and time-relationships between DNA adduct formation and mutagenesis by NDMA, λ*lac*Z transgenic mice were treated with single or multiple doses and adducts and mutations measured at various times. Attention was focused mainly on the liver and lung (known targets for NDMA carcinogenesis), as well as the spleen and the bone marrow in view of the high proliferation rate of these tissues. Additional tissues in which adducts were measured include white blood cells (WBC) and the brain.

DNA adducts. The kinetics of adduct formation and repair was examined in animals treated with single doses of 5 mg/kg or 10 mg/kg NDMA. As can be seen in Figures 1 and 2, the liver showed by far the highest adduct levels, followed by the lung, spleen, WBC, bone marrow and brain. Six h after treatment with 5 mg/kg, the levels of O^6- and *N*7-meG had already reached maximal values, thereafter decreasing with half-lives of 20–25 h and 35–55 h, respectively, depending on the tissue. Whereas no O^6-meG could be detected 7 days after treatment, the more slowly repaired adduct *N*7-meG was still detectable in liver DNA at levels substantially higher than background even 14 days post-treatment. Significant depletion of AGT was observed 6 h after treatment in all tissues examined, followed by slow return to full activity within 7–14 days.

Animals were also treated with multiple doses of NDMA, consisting of 1 mg/kg NDMA every 24 h for 10 days. Tissues were collected 6 h after the 1st, 6th and 10th treatments, and DNA adducts and AGT measured. As can be seen in Figure 3, the tissue distribution of adducts was similar to that seen after single doses, with the liver showing at least one order of magnitude higher adduct accumulation than the lung and the other tissues. In the liver, WBC and spleen, adducts approached steady-state levels by day 10. Although no O^6-meG could be detected in the bone marrow and brain at any time point examined, significant AGT depletion was observed in these tissues, implying that O^6-meG accumulation at levels below the detection limit had probably occurred.

Mutant frequencies. Mutant frequencies were measured in the liver, lung, spleen and bone marrow 7, 14, 28 and 49 days after 5 mg/kg NDMA and 14 and 28 days after 10 mg/kg NDMA. As can be seen in Table I, after 5 mg/kg NDMA a significant increase in MF occurred in the liver, where the MF had doubled by day 7 and remained practically unchanged until day 49. A small but statistically significant ($p = 0.037$) increase also occurred in the spleen 7 days after treatment. An analogous picture was obtained after 10 mg/kg NDMA (Table II), with the MF increasing substantially in the liver, where it almost quadrupled after 14 days and remained constant up to 28 days, and to a smaller but statistically significant degree ($p = 0.008$) in the spleen 28 days post-treatment. No evidence of an increase in the MF was obtained for the bone marrow and lung.

Mutant frequencies were also measured 14 and 28 days

Table I. Mutant frequencies (mean, sd) in λ*lac*Z transgenic mice after a dose of 5 mg/kg NDMA

	MF (per 10^6 pfu) (SD)			
Time (days)	Liver	Bone marrow	Lung	Spleen
0	59 (8)	28 (6)	63 (23)	35 (8)
7	97[b] (25)	31 (10)	61 (18)	65 (21)
14	109[b] (21)	41[a] (4)	56 (12)	43 (6)
28	106[b] (21)	28 (8)	56 (13)	25 (10)
49	110[c] (8)	32 (11)	71 (26)	35 (6)

Data from 4 mice, unless otherwise indicated. For experimental details, see Materials and methods.
[a]Data from 3 mice.
[b]$p < 0.05$.
[c]$p < 0.001$ (*t*-test).

Table II. Mutant frequencies (mean, sd) in λ*lac*Z transgenic mice after a dose of 10 mg/kg NDMA

	MF (per 10^6 pfu) (SD)			
Time (days)	Liver	Bone marrow	Lung	Spleen
0 (control)	57 (12)	40 (12)	54 (7)	40 (6)
14	219[a] (79)	47 (13)	61 (6)	38 (7)
28	205[a] (81)	48[c] (7)	56 (6)	63[b] (10)

Data from 4 mice, unless otherwise indicated; two controls were analysed on day 14 and two on day 28. For experimental details, see Materials and methods.
[a]$p < 0.05$.
[b]$p < 0.01$ (*t*-test).
[c]Data from 3 mice.

Table III. Mutant frequencies (mean, SD) in λ*lac*Z transgenic after 10 daily doses of 1 mg/kg NDMA

	MF (per 10^6 pfu)			
Time (days)	Liver	Bone marrow	Lung	Spleen
0 (control)	64 (29)	33 (11)	54 (7)	40 (6)
14	102 (24)	32 (7)	58 (9)	35 (6)
28	137[a] (33)	31 (10)	64 (8)	69 (26)

Data from 4 mice; two controls were analysed on day 14 and two on day 28. For experimental details, see Materials and methods.
[a]$p < 0.05$ (*t*-test).

after completion of the 10-day treatment with 1 mg/kg/day NDMA (Table III). An increase was observed in the liver, which became significant ($p = 0.019$) 28 days post-treatment. As was the case after single doses, a small (1.7-fold) increase was observed in the spleen on day 28. This increase is significant ($p = 0.029$) when compared with the day 14 value, although it just failed to reach statistical significance when compared to the control animal value ($p = 0.06$).

Sequence-characterisation of mutations

Spontaneous mutations. Fourty-three mutant plaques obtained from the livers of four control mice were selected at random and sequence-characterised. In four of these mutants, multiple mutations were observed. One mutation (a –C deletion at nucleotide position 109) was observed twice. Of the 47 mutations thus analysed, the majority (70%) consisted of single-base substitutions, including 41% transitions and 29% transversions (Tables IV and V). The most frequent type of mutation was GC→AT transitions (28%), including 5/13 at CpG and 4/13 at GpG dinucleotides. In addition, all possible single-base substitutions were observed, as well as eight single-base deletions, two single-base insertions, two large deletions and one three-base pair insertion.

Mutations in NDMA-treated mice. Fourty-three randomly selected mutants obtained from the livers of four mice 28 days after treatment with 10 mg/kg NDMA were sequenced. In four cases, multiple mutations were found. Of the 47 mutations thus analysed, 64% were single-base substitutions, consisting predominantly of transitions (58%) and a few transversions (6%) (Tables V and VI). In addition, seven single-base deletions, one single-base insertion, eight larger deletions of 4–100-bases and one 5-base insertion were found. Taking into account that these mutants were derived from a treatment which caused a 3.6-fold increase in MF relative to the controls, the observed mutation spectrum was corrected for the probable contribution of spontaneous mutations (Table V). The only types of mutations in the calculated net mutation spectrum which were significantly (χ^2 test) increased were GC→AT transitions and single or multiple base deletions.

Discussion

In this study, quantitative and qualitative data on the induction by NDMA of methylated DNA adducts and gene mutations in λ*lac*Z transgenic mice have been obtained. The liver was the main target for adduct induction, accumulating ~10–30 times more adducts than the lung (tissue with the next most abundant adducts). The liver was also the primary target for mutagenesis. Single doses of 5 mg/kg or 10 mg/kg NDMA resulted in clear increases in liver MF within 7 days, with no significant further change up to 49 days after treatment, indicating that the process of conversion of premutagenic DNA lesions into mutations in this tissue was complete within 7 days. By this time, O^6-meG (the most potent premutagenic lesion induced by NDMA) was no longer detectable in DNA. Fourteen days post-treatment, *N*7-meG was still detectable in liver DNA at levels (20.3 fmol/μg DNA) which were greatly reduced but still significantly higher than in control animals. Based on the observed rate of loss of *N*7-meG, the levels of this adduct in liver DNA 28 days post-treatment are likely to have been of the order of 1 fmol/μg, comparable to that observed in the liver of untreated animals (Figure 1).

Table VII shows that the yield of liver adducts per unit dose (mg/kg NDMA) 6 h after treatment with 5 mg/kg or 10 mg/kg NDMA is constant, whereas it is significantly reduced after a dose of 1 mg/kg. While adduct levels at a specific time-point constitute a useful measure of the amount of the genotoxic

Table IV. Liver mutations in control λ*lac*Z transgenic mice

Sequence alteration	Region	Position	Target sequence	Amino acid alteration
GC→AT	α	436	AGACG**C**GAAT	arg→STOP
GC→AT	β	595	GAGT**G**ACGGC	asp→asn
GC→AT	β	1073	TC**G**AGGCGTT	arg→gln
GC→AT	β	1114	CAG**G**TCATGG	val→ile
GC→AT	β	1373	GA**G**TGTGATC	ser→asn
GC→AT	β	1492	C**G**ACACCACG	asp→asn
GC→AT	β	1543	AA**G**ACCAGCC	asp→asn
GC→AT	β	1561	T**G**TGCCGAAA	val→met
GC→AT	β	1676	ATACT**G**GCAG	trp→STOP
GC→AT	β	1688	GCGTTTC**G**TC	arg→his
GC→AT	β	1708	TTTACAG**G**GC	gln→ser
GC→AT	ω	2153	CG**G**GCACATC	gly→glu
GC→AT	ω	2659	GGCG**C**GGA	arg→STOP
AT→GC	β	625	TATG**T**GGCG	trp→arg
AT→GC	β	913	CG**A**AACTGTG	lys→gln
AT→GC	β	1202	C**A**TCCGCTGT	his→pro
AT→GC	β	1232	T**A**CGGCCTGT	tyr→ser
AT→GC	β	1510	CACCGAT**A**TT	ile→leu
AT→GC	ω	2408	GTAAGTG**A**AG	glu→ala
GC→TA	α	187	GGC**G**AATGG	glu→STOP
GC→TA	β	994	GCA**G**AAGCC	glu→STOP
GC→TA	β	1722	TG**G**GACTGGG	trp→cys
GC→TA	β	1739	GT**C**GCTGAT	ser→STOP
GC→TA	ω	1823	CT**G**TATGAAC	cys→phe
GC→TA	ω	2266	AGCGC**C**TGG	glu→trp
GC→TA	ω	2493	AGTG**C**ACGG	cys→STOP
GC→CG	β	463	CG**G**CGTTT	ala→pro
GC→CG	β	1627	GC**G**AATAC	glu→gln
GC→CG	ω	2215	CGCC**G**CGTCC	ala→pro
AT→CG	β	905	GTCG**A**AAACC	glu→ala
AT→TA	β	548	TTTT**T**ACGC	leu→STOP
AT→TA	β	899	ACGTCTGA**A**C	asn→ile
AT→TA	β	1438	GTATCGC**T**GG	trp→arg
–C	α	109	ACAT**C**CCCCT	
–G	β	479	GGT**G**CAAC	
–G	β	756	CAGAT**G**TGC	
–C	β	759	GTG**C**GGCGA	
–G	β	883	GC**G**TCACAC	
–19bp	β	1044–1062	CTGCT...TTGC	
–A	β	1144	GAT**A**TCCTGC	
–G	β	1179	GT**G**CGCTG	
–52bp	β	1502–1553	ACGG...CCCG	
–T	β	1656	AGTCT**T**GGCG	
+ATA		1512–1513	TT**ATA**ATTTGCC	
or	β	or	or	
+TAA		1513–1514	TTA**TAA**TTTGCC	
+C	β	1734–1735	GGAT'CAGTC	
+T	ω	1849–1850	ACC'GCACG	

Table V. Summary of mutations observed in control and NDMA-treated λ*lac*Z transgenic mice

	Control		NDMA-treated		
	Observed	%	Observed	%	Corrected for contibution of spontaneous (%)[b]
Transition					
GC→AT	13 (5)[a]	28	24 (5)[a]	51	60 ($p < 0.001$)
AT→GC	6	13	3	6	
Transversions					
GC→TA	7	15	–		
GC→CG	3	6	2	4	
TA→AT	3	6	–		
TA→GC	1	2	1	2	
Deletions					
–1	9	19	7	15	14 ($p = 0.031$)
>–1	2	4	8	17	22 ($p = 0.003$)
Insertions	3	7	2	4	
Total	47	100	47	100	

[a]In brackets, mutations within 5′ CpG dinucleotides.
[b]Only mutations calculated to be statistically significantly increased (χ^2 test) by treatment are shown.

damage occurring after a given dose, a more satisfactory quantitative measure of the biologically effective extent of DNA damage occurring in a given tissue would be provided by the area under the corresponding adducts–time curve. Calculation of the areas under the curve over the period 0–14 days after 5 mg/kg shows that, for different tissues, they correlate closely with the corresponding 6 h levels for both O^6- and *N*7-meG ($r^2 = 1.00$; $p \leqslant 0.01$) (results not shown), indicating that the 6 h measurements reflect accurately the area under the curve over a wide range of tissue-specific adduct concentrations and repair capacities. Given that almost complete depletion of AGT was observed after both 5 mg/kg and 10 mg/kg NDMA, it is likely that the same correlation holds for the dose of 10 mg/kg NDMA (for which the available data do not permit the direct calculation of the corresponding areas under the adducts curve). Thus, the time-integrated concentration of the two major DNA adducts appears to be linearly related to the administered NDMA dose in the 5–10 mg/kg range. In contrast, the corresponding MF 14 or 28 days post-treatment shows clearly superlinear relationships with the dose (p for linearity >0.12) and the 6 h adduct levels (Table VII). Characteristically, the number of net induced mutants (post-NDMA minus spontaneous) 28 days after 10 mg/kg was 148 per 10^6 pfu, >3-fold greater than that induced by 5 mg/kg (47 per 10^6 pfu). This greater than linear increase of MF is likely to be the result of increased liver cell proliferation caused by the hepatotoxicity of 10 mg/kg NDMA (33,54–56).

During 10 daily administrations of 1 mg/kg NDMA, O^6- and *N*7-meG accumulated steadily in liver DNA, their 6 h adduct levels approaching a constant value by the end of this treatment period. Consequently the biologically effective concentration of each adduct throughout the dosing period, as reflected by the area under the curve, cannot be accurately estimated from the available data, since following each administration of NDMA there would have occurred a rapid increase in adduct levels followed by repair. However, the less extensive depletion of AGT and the consequent lower yield of O^6-meG per unit dose caused by 1 mg/kg NDMA (Figure 3) imply that the area under the adducts curve for O^6-meG during the split-dose regimen was less than that following a single administration of 10 mg/kg. As shown in Table VII, 28 days after the end of the split-dose regimen the net induced MF in the liver was 73 per 10^6 pfu, less than half of that caused by the same total dose of 10 mg/kg administered at once. Since no significant decrease in MF occurred up to 49 days after 5 mg/kg NDMA, the lower mutagenic efficiency of split dosing cannot be attributed to mutant loss, but was probably related to the lower effective concentration of O^6-meG and the more limited toxicity-induced cell proliferation. While no systematic study of the effects of dose and time on toxicity-induced cell proliferation has been reported, it is noted that treatment of C3H mice with NDMA dissolved in their drinking water at a concentration of 30 ppm (resulting in a daily dose of ~5 mg/kg) for 16 days gave rise to a significant increase in hepatocyte proliferation, whereas no increase was observed in animals exposed to 3-fold lower concentration of NDMA (57). Furthermore, Doolittle *et al.* (54) reported that seven daily treatments of CD-1 mice with 4 mg/kg NDMA gave rise to a toxicity-associated increase in hepatocyte replication, whereas a similar treatment with 2 mg/kg had no detectable effect. Finally, Suzuki *et al.* (33) recently reported that five daily oral doses of 1 mg/kg NDMA did not induce any significant cell proliferation in hepatocytes of Big Blue (C57Bl/C6) mice. Based on these data, it appears that little if any hepatocyte proliferation would be induced by 10 daily doses of 1 mg/kg NDMA as employed in our study, whereas it would have been after the highest single dose employed, i.e. 10 mg/kg.

Our finding that 10 mg/kg NDMA is more effective in inducing liver mutations when administered as a single dose than when split over 10 days contrasts with the recent report of Suzuki *et al.* (33) who reported that five daily doses of 1 mg/kg NDMA were more mutagenic in the liver of 8-week old male Big Blue mice than a single dose of 5 mg/kg. In view of the similarity in mouse strain (except for the transgene genotype), age and sex employed in that study and ours, this difference is unexpected and we have no satisfactory explanation for it, other than speculate that the more extended period of exposure in our study may have resulted in different

Table VI. Liver mutations in NDMA-treated λ*lac*Z transgenic mice

Sequence alteration	Region	Position	Target sequence	Amino acid alteration
GC→AT	α	85	ACC<u>C</u>AACTT	gln→STOP
GC→AT	α	217	GCACCA<u>G</u>AAG	glu→lys
GC→AT	α	237	AGCTG<u>G</u>CTG	trp→STOP
GC→AT	α	403	AT<u>G</u>TTGATGA	val→ile
GC→AT	β	556	GCGCC<u>G</u>GAGA	gly→arg
GC→AT	β	619	AAGATCAG<u>G</u>AT	asp→asn
GC→AT	β	646	TTTTC<u>C</u>GTGA	arg→cys
GC→AT	β	801	TTATG<u>G</u>CAG	trp→STOP
GC→AT	β	955	GGTG<u>G</u>TTGAA	val→ile
GC→AT	β	1090	CAC<u>G</u>AGCAT	glu→lys
GC→AT	β	1177	AACGCC<u>G</u>TGCG	val→met
GC→AT	β	1249	TGTGGTG<u>G</u>ATG	asp→asn
GC→AT	β	1279	GGCATG<u>G</u>TGCC	val→met
GC→AT	β	1303	AT<u>G</u>ATCCGCG	asp→asn
GC→AT	β	1468	CCGCCCG<u>G</u>TG	val→met
GC→AT	β	1537	CGCGTG<u>G</u>ATG	asp→asn
GC→AT	β	1645	GATG<u>G</u>GTAAC	gly→ser
GC→AT	β	1700	GTATCCCC<u>G</u>TTT	arg→his
GC→AT	β	1709	CAGG<u>G</u>CGGC	gly→asp
GC→AT	ω	2045	CAAG<u>G</u>TAAAC	gly→asp
GC→AT	ω	2317	TCA<u>C</u>AGATG	gln→STOP
GC→AT	ω	2772	GCTG<u>G</u>GATCT	trp→STOP
GC→AT	ω	2837	GCTGCG<u>G</u>GAC	gly→glu
GC→AT	ω	2871	CAGTG<u>G</u>CGC	trp→STOP
AT→GC	β	457	GGCGTT<u>A</u>ACT	asn→asp
AT→GC	β	809	GCAGGGTG<u>A</u>A	glu→gly
AT→GC	ω	2638	AGTGGCG<u>A</u>GC	ser→gly
GC→CG	ω	2539	CCGCTCAC<u>G</u>CG	val→leu
GC→CG	ω	2840	GGGA<u>C</u>GCGC	thr→arg
TA→GC	β	1168	GAACAAC<u>T</u>TT	phe→val
–G	α	229	GCCG<u>G</u>AAAG	
–C	β	1182	G<u>C</u>TGTTCGCA	
–34bp	β	1243–1276	TAT...TGGT	
–7bp	β	1430–1437	CGCT...TGGA	
–T	β	1509	GCCACCGA<u>T</u>A	
–5bp	β	1605–1609	GAGA...GCCCG	
–7bp	β	1644–1650	GAT...AGT	
–A	β	1744	CTG<u>A</u>TTAAAT	
–4bp	ω	1830–1833	TGAA...CTGG	
–11bp	ω	1995–2005	TGGA...TGGCA	
–5bp	ω	2123–2127	GTGC...AACGC	
–T	ω	2357	CCGC<u>T</u>GCGC	
–100bp	ω	2475–2574	CCGA...CGGAA	
–C	ω	2722	GGG<u>C</u>CGCAA	
–A	ω	2909	AGTCAACAG	
+T	β	1163	GAA'CAACTTT	
+5T	ω	2255–2260	GA'T'T'T'T'T'GC	

Table VII. Summary data on the induction of DNA adducts and mutations (mean; SD) by different doses of NDMA

Dose	O^6-meG[b]/ total dose	$N7$-meG[b]/ total dose	Induced MF[c]	Induced MF/ total dose
1 mg/kg[a]	9.9 (0.9)	104 (22)		
5 mg/kg	16.6 (1.6)	130 (5)	47 (29)	9.4 (5.8)
10 mg/kg	17.6 (1.0)	159 (15)	148 (93)	14.8 (9.3)
10 × 1 mg/kg	1.35 (0.13)	17.8 (4.7)	73 (62)	7.3 (6.2)

[a]First of 10 daily doses of 1 mg/kg.
[b]6 h post-treatment.
[c]28 days post-treatment.

cell replication kinetics in specific subpopulations of liver cells which constitute targets for NDMA mutagenesis (58). We note, in this respect, that Mirsalis *et al.* (35) failed to induce mutations after five daily treatments of 2 mg/kg NDMA in 6-week old *lac*I mice, although they did report a positive response in 3-week old mice given the same dosage. An additional point of contrast of our results with those of Suzuki *et al.* (33) concerns our failure to observe induction of lung mutations by NDMA. On the other hand, in agreement with these authors, we too observed no NDMA-induced mutagenesis in the bone marrow. The levels of O^6-meG in this tissue after 10 mg/kg NDMA were ~200-fold lower than in the liver (Figure 2) while they were below the limit of detection after 10 daily doses of 1 mg/kg (Figure 3). While NDMA causes clastogenic damage in the bone marrow (micronucleus induction and sister-chromatid exchanges) after animal treatment at very high, toxic doses, it appears to have marginal clastogenic effects at doses in the range of 5–10 mg/kg as employed in the present study (33,59,60).

Of the tissues examined for mutagenesis in our study, the spleen was the only one, in addition to the liver, for which consistent evidence of a small mutagenic effect was obtained. Small (<2-fold) but statistically significant increases in spleen MF were observed 7 days after 5 mg/kg and 28 days after 10 mg/kg NDMA, while a small increase which just failed to reach statistical significance was also observed 28 days after the end of 10 daily administrations of 1 mg/kg NDMA. Although examination of larger groups of animals would be required to obtain conclusive support for the significance of such small increases in MF, their consistent occurrence after all three dosing regimens and only in this tissue suggests that NDMA may indeed have a mutagenic effect on the spleen, despite the accumulation in this tissue of ~40–100-fold fewer adducts than in the liver. We note that the spleen is particularly susceptible to methylating agent mutagenesis, exhibiting the highest levels of mutagenesis after administration of a single dose of MNU, a direct-acting methylating agent which, in contrast to NDMA, causes significant methylation in many tissues, including the spleen (61).

Our observations on the spectrum of spontaneous mutations in the liver of *lac*Z transgenic mice confirm and extend those reported by Douglas *et al.* (62). Based on a total of 47 mutations sequenced, we found that GC→AT transitions are the most frequent spontaneous mutations, a large proportion of them being concentrated at CpG dinucleotides. We also observed all the other possible single-base substitutions, as well as a substantial number of frameshift mutations, including eight single-base and two larger deletions and three insertions.

We sequenced 47 liver mutations isolated 28 days after treatment with a single dose of 10 mg/kg NDMA, a treatment which resulted in a 3.6-fold increase in MF. Correction of the observed mutation spectrum for the likely contribution of spontaneous mutagenesis revealed that NDMA induced mainly GC→AT transitions (~60%) and deletions. Sequencing of a limited number of liver mutations induced in *lac*I transgenic mice by five daily doses of 4 mg/kg NDMA was reported to reveal 8/10 GC→AT transitions, one TA→GC transversion and one single-base insertion (35). The proportion of GC→AT mutations obtained by us from the analysis of a much larger number of mutations is comparable to that reported above, as well as that caused by MNU in mammalian cells (29,63–65), and reflects the important role of direct miscoding by O^6-meG during DNA replication. On the other hand, this proportion is significantly lower than that observed in *E.coli in vitro* or in

combined *in vivo*/*in vitro* systems (30,32,35), a difference which may reflect the high probability of conversion into mutations of a short-lived, highly mutagenic lesion such as O^6-meG in rapidly proliferating bacterial cells. Approximately 63% of GC→AT mutations observed in our study were at 5′GpG sites, in agreement with the known sequence specificity of S_N1-type methylating agents (64,65).

The finding that approximately one third of NDMA-induced mutations involved deletion of one or more bases is unexpected. While the confounding of the mutation spectrum in NDMA-treated animals by spontaneous mutations does not permit the assignment of individual mutations to NDMA, it is statistically probable that most of the observed deletions of one or a few (up to 11) bases were caused by NDMA. As discussed above, the reported mutation spectrum of MNU in mammalian cells includes a significant proportion of non-GC→AT mutations. Furthermore, it has been noted that NDMA and its direct-acting analogue N-methyl-N-acetoxymethylnitrosamine appear to induce in *E.coli* non-GC>AT mutations somewhat more frequently than MNU or MNNG (30,32,66). However, such mutations consisted primarily of other types of single-base substitutions, deletions being exceedingly rare among them. The only case where a significant induction of deletions by MNU, MNNG and NDMA has been reported concerns mutagenesis in the vermillion gene of postmeiotic male germ cells of *Drosophila melanogaster*, where these methylating agents caused substantial numbers of deletions of a few to a few thousand base-pairs (but no single-base deletions) (67). This observation demonstrates that, in the presence of appropriate host conditions, methylation damage of DNA includes lesions capable of leading to deletions. We note, in this respect, that we have recently observed that the methylating cancer chemotherapeutic drug procarbazine also causes a substantial number of small deletions in the bone marrow of λ*lac*Z transgenic mice (68). Some methylated DNA adducts (especially *N*3-methyladenine, but also O^6-meG) can act as full or partial blocks of DNA replication (69), thus possibly leading to strand misalignment and consequent deletions, especially small deletions. It is also possible that base excision repair of lesions at ring nitrogens (including the abundant adduct *N*7-meG) might give rise to deletions if not properly completed (70). Another possibility is that abortive mismatch repair of O^6-meG:T pairs through repeated cycles of excision and resynthesis of the strand opposite the adduct (71,72), believed to be primarily a cause of cell death, may also act as a source of deletions in surviving cells. Finally, it is possible that NDMA may generate one or more genotoxic intermediates different from that produced by MNU, something for which there exists additional evidence (73,74). One such possible metabolite is formaldehyde produced in situ during the oxidative metabolism of NDMA. Formaldehyde is known to damage DNA, inducing primarily protein-DNA crosslinks which can give rise to deletions or other major changes in the DNA sequence (75,76). Indeed, formaldehyde causes, in addition to base substitutions, large deletions and insertions (77,78), and there is evidence that their relative proportions can vary depending on the dose employed. It is important to have in mind, in this respect, that during NDMA's metabolism formaldehyde is produced in equimolar proportion to the methylating intermediate within the cell and in close proximity to the nucleus. Evidence that formaldehyde produced during metabolism may mediate mutagenicity has recently been provided for another chemical, methylene chloride (79).

Summarising, we have found that the extent of NDMA-induced mutagenesis in mouse liver increases disproportionately to dose intensity and to DNA adduct induction in the dose region 1–10 mg/kg. This suggests that toxicity-induced liver cell proliferation may play an important role in determining NDMA mutagenesis in the liver and that extrapolation of NDMA-mediated mutagenic effects to low dose levels should not be based on the assumption of dose linearity, even if dose is expressed in terms of DNA damage. At the highest dose examined (10 mg/kg), in addition to GC→AT mutations, NDMA induces a significant proportion of deletions of one or a few bases. The elucidation of the molecular mechanism of these mutations may assist in the interpretation of mutation spectra in cancer genes of human cancers in whose etiology a possible role of NDMA is suspected.

Acknowledgements

The valuable technical assistance of Margarita Bekyrou, Stella Kaila and Irene Kolonti is acknowledged. This work was financially supported by the European Union under contract no. ENV5V-CT92-0224.

References

1. Anonymous (1978) N-Nitrosodimethylamine. *IARC Monographs on the Evaluation of the Carcinogenic Risks of Chemicals to Humans No. 17*. International Agency for Research on Cancer, Lyon.
2. Magee,P.N. (1989) The experimental basis for the role of nitroso compounds in human cancer. *Cancer Surv.*, **8**, 207–239.
3. Hotckiss,J.H. (1989) Preformed N-nitroso compounds in foods and beverages. *Cancer Surv.*, **8**, 295–321.
4. Hecht,S.S. and Hoffmann,D. (1989) The relevance of tobacco-specific nitrosamines to human cancer. *Cancer Surv.*, **8**, 273–294.
5. Pignatelli,B., Malaveille,C., Rogatko,A., Hautefeuille,A., Thuillier,P., Munoz,N., Moulinier,B., Berger,F., Demontclos,H., Lambert,R., Correa,P., Ruiz,B., Sobala,G.M., Schorah,C.J., Axon,A.T.R. and Bartsch,H. (1993) Mutagens, N-nitroso compounds and their precursors in gastric juice from patients with and without precancerous lesions of the stomach. *Eur. J. Cancer*, **29A**, 2031–2039.
6. Xu,G.P. and Reed,P.I. (1993) N-nitroso compounds in fresh gastric juice and their relation to intragastric pH and nitrite employing an improved analytical method. *Carcinogenesis*, **14**, 2547–2551.
7. Marletta,M.A. (1988) Mammalian synthesis of nitrite, nitrate, nitric oxide and N-nitrosating agents. *Chem. Res. Toxicol*., **1**, 249–257.
8. Leaf,C.D., Wishnok,J.S. and Tannenbaum,S.R. (1989) Mechanisms of endogenous nitrosation. *Cancer Surv.*, **8**, 323–334.
9. Tannenbaum,S.R. (1980) A model for estimation of human exposure to endogenous N-nitrosodimethylamine. *Oncology*, **37**, 232–235.
10. Kyrtopoulos,S.A., Souliotis,V.L., Chhabra,S.K. and Anderson,L.M. (1996) DNA damage studies related to the assessment of the role of N- nitroso compounds in human cancer. *Eur. J. Cancer Prev.*, **5** (Suppl. 1), 109–114.
11. Bartsch,H. (1991) N-nitroso compounds and human cancer: where do we stand? In O'Neill,I., Chen,J. and Bartsch,H. (eds) *Relevance to Human Cancer of N-Nitroso Compounds*, Tobacco *Smoke and Mycotoxins*. IARC Scientific Publications No. 105, Lyon, pp. 1–10.
12. Norman,M.A., Holly,E.A., Ahn,D.K., Preston-Martin,S., Mueller,B.A. and Bracci,P.M. (1996) Prenatal exposure to tobacco smoke and childhood brain tumours: Results from the United States West Coast childhood brain tumour study. *Cancer Epidemiol. Biomarkers Prev.*, **5**, 127–134.
13. Preston-Martin,S., Pogoda,J.M., Mueller,B.A., Holly,E.A., Lijinsky,W. and Davis,R.L. (1996) Maternal consumption of cured meats and vitamins in relation to pediatric brain tumors. *Cancer Epidemiol. Biomarkers Prev.*, **5**, 599–606.
14. Rogers,M.A., Vaughan,T.L., Davis,S. and Thomas,D.B. (1995) Consumption of nitrate, nitrite, and nitrosodimethylamine and the risk of upper aerodigestive tract cancer. *Cancer Epidemiol. Biomarkers Prev.*, **4**, 29–36.
15. Mostafa,M.H., Helmi,S., Badawi,A.F., Tricker,A.R., Spiegelhalder,B. and Preussmann,R. (1994) Nitrate, nitrite and volatile N-vitroso compounds in the urine of schistosoma haematobium and schistosoma mansoni infected patients. *Carcinogenesis*, **15**, 619–625.
16. Parkin,D.M., Srivatanakul,P., Khlat,M., Chenvidhya,D., Chotiwan,P., Insiripong,S., L'Abbe,K.A. and Wild,C.P. (1991) Liver cancer in Thailand. I. A case-control study of cholangiocarcinoma. *Int. J. Cancer*, **48**, 323–328.

17. Preston-Martin,S. (1991) Gliomas and meningiomas in men in Los Angeles county: investigation of exposures to N-nitroso compounds. In O'Neill,I., Chen,J. and Bartsch,H. (eds) *Relevance to Human Cancer of N-Nitroso Compounds, Tobacco Smoke and Mycotoxins*. IARC Scientific Publications No. 105, Lyon, pp. 197–203.
18. Bartsch,H., Ohshima,H., Pignatelli,B. and Calmels,S. (1989) Human exposure to endogenous N-nitroso compounds: quantitative estimates in subjects at high risk for cancer of the oral cavity, oesophagus, stomach and urinary bladder. *Cancer Surv.*, **8**, 335–362.
19. Yoo,J.S.H., Ishizaki,H. and Yang,C.S. (1990) Roles of cytochrome P450IIE1 in the dealkylation and denitrosation of N-nitrosodimethylamine and N-nitrosodiethylamine in rat liver microsomes. *Carcinogenesis*, **11**, 2239–2243.
20. Beranek,D.T. (1990) Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. *Mutat. Res*., **231**, 11–30.
21. Takeshita,M. and Eisenberg,W. (1994) Mechanism of mutation on DNA templates containing synthetic abasic sites: Study with a double strand vector. *Nucleic Acids Res*., **22**, 1897–1902.
22. Swenberg,J.A., Bedell,M.A., Billings,K.C., Umbenhauer,D.R. and Pegg,A.E. (1982) Cell-specific differences in O^6-alkylguanine repair activity during continuous exposure to carcinogen. *Proc. Natl Acad. Sci. USA*, **79**, 5499–5502.
23. Fong,L.Y., Bevill,R.F., Thurmon,J.C. and Magee,P.N. (1992) DNA adduct dosimetry and DNA repair in rats and pigs given repeated doses of procarbazine under conditions of carcinogenicity and human cancer chemotherapy respectively. *Carcinogenesis*, **13**, 2153–2159.
24. Zaidi,N.H., Allay,E., Ayi,T.C., Li,B.F.L., Dumenco,L.L., Sy,M.S. and Gerson,S.L. (1995) The immature thymocyte is protected from N-methylnitrosourea-induced lymphoma by the human MGMT-CD2 transgene. *Carcinogenesis*, **16**, 1047–1053.
25. Zaidi,N.H., Pretlow,T.P., O'Riordan,M.A., Dumenco,L.L., Allay,E. and Gerson,S.L. (1995) Transgenic expression of human MGMT protects against azoxymethane-induced aberrant crypt foci and G→A mutations in the K-*ras* oncogene of mouse colon. *Carcinogenesis*, **16**, 451–456.
26. Tan,H.B., Swann,P.F. and Chance,E.M. (1994) Kinetic analysis of the coding properties of O^6-methylguanine in DNA — the crucial role of the conformation of the phosphodiester bond. *Biochemistry*, **33**, 5335–5346.
27. Pauly,G.T., Hughes,S.H. and Moschel,R.C. (1995) Mutagenesis in *Escherichia coli* by three O^6-substituted guanines in double-stranded or gapped plasmids. *Biochemistry*, **34**, 8924–8930.
28. Pletsa,V., Troungos,C., Souliotis,V.L. and Kyrtopoulos,S.A. (1994) Comparative study of mutagenesis by O^6-methylguanine in the human Ha-*ras* oncogene in *E.coli* and *in vitro*. *Nucleic Acids Res*., **22**, 3846–3853.
29. Jansen,J.G., Mohn,G.R., Vrieling,H., van Teijlingen,C.M., Lohman,P.H. and van Zeeland,A.A. (1994) Molecular analysis of hprt gene mutations in skin fibroblasts of rats exposed *in vivo* to N-methyl-N-nitrosourea or N-ethyl-N- nitrosourea. *Cancer Res*., **54**, 2478–2485.
30. Jiao,J., Glickman,B.W., Anderson,M.W. and Zielinska,M. (1993) Mutational specificity of N-nitrosodimethylamine: comparison between *in vivo* and *in vitro* assays. *Mutat. Res*., **301**, 27–31.
31. Yang,J.L., Lin,J.G., Hu,M.C. and Wu,C.W. (1993) Mutagenicity and mutational spectrum of N-methyl-N′-nitro-N-nitrosoguanidine in the hprt gene in G1-S and late S-phase of diploid human fibroblasts. *Cancer Res*., **53**, 2865–2873.
32. Horsfall,M.J., Zeilmaker,M.J., Mohn,G.R. and Glickman,B.W. (1989) Mutational specificities of environmental carcinogens in the *lac*I gene of *Escherichia coli*. II: A host-mediated approach to N-nitroso-N,N-dimethylamine and endogenous mutagenesis *in vivo*. *Mol. Carcinog*., **2**, 107–115.
33. Suzuki,T., Itoh,T., Hayashi,M., Nishikawa,Y., Ikezaki,S., Furukawa,F., Takahashi,M. and Sofuni,T. (1996) Organ variation in the mutagenicity of dimethylnitrosamine in Big Blue mice. *Environ. Mol. Mutagen*., **28**, 348-353.
34. Tinwell,H., Lefevre,P.A. and Ashby,J. (1994) Response of the Muta mouse *lac*Z/galE-transgenic mutation assay to DMN: comparisons with the corresponding Big Blue (*lac*I) responses. *Mutat. Res*., **307**, 169–173.
35. Mirsalis,J.C., Provost,G.S., Matthews,C.D., Hamner,R.T., Schindler,J.E., O'Loughlin,K.G., Macgregor,J.T. and Short,J.M. (1993) Induction of hepatic mutations in *lac*I transgenic mice. *Mutagenesis*, **8**, 265–271.
36. Shephard,S.E., Lutz,W.K. and Schlatter,C. (1994) The *lac*I transgenic mouse mutagenicity assay: quantitative evaluation in comparison to tests for carcinogenicity and cytogenetic damage *in vivo*. *Mutat. Res*., **306**, 119–128.
37. Matsumoto,K., Iwase,T., Hirono,I., Nishida,Y., Iwahori,Y., Hori,T., Asamoto,M., Takasuka,N., Kim,D.J., Ushijima,T., Nagao,M. and Tsuda,H. (1997) Demonstration of *ras* and *p53* gene mutations in carcinomas in the forestomach and intestine and soft tissue sarcomas induced by N-methyl-N-nitrosourea in the rat. *Jpn. J. Cancer Res*., **88**, 129–136.
38. Chang,K.W., Lin,S.C., Koos,S., Pather,K. and Solt,D. (1996) *p53* and Ha-*ras* mutations in chemically induced hamster buccal pouch carcinomas. *Carcinogenesis*, **17**, 595–600.
39. Wang,D., Weghorst,C.M., Calvert,R.J. and Stoner,G.D. (1996) Mutation in the *p53* tumor suppressor gene in rat esophageal papillomas induced by N-nitrosomethylbenzylamine. *Carcinogenesis*, **17**, 625–630.
40. Newcomb,E.W., Bayona,W. and Pisharody,S. (1995) N-methylnitrosourea-induced Ki-*ras* codon 12 mutations: early events in mouse thymic lymphomas. *Mol. Carcinog*., **13**, 89–95.
41. Ronai,Z.A., Gradia,S., Peterson,L.A. and Hecht,S.S. (1993) G→A transitions and G→T transversions in codon 12 of the Ki-*ras* oncogene isolated from mouse lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and related DNA methylating and pyridyloxobutylating agents. *Carcinogenesis*, **14**, 2419–2422.
42. Devereux,T.R., Anderson,M.W. and Belinsky,S.A. (1991) Role of *ras* protooncogene activation in the formation of spontaneous and nitrosamine-induced lung tumors in the resistant C3H mouse. *Carcinogenesis*, **12**, 299–303.
43. Sukumar,S., Armstrong,B., Bruyntjes,J.P., Leav,I. and Bosland,M.C. (1991) Frequent activation of the Ki-*ras* oncogene at codon 12 in N-methyl-N-nitrosourea-induced rat prostate adenocarcinomas and neurogenic sarcomas. *Mol. Carcinog*., **4**, 362–368.
44. Warren,W., Biggs,P.J., el-Baz,M., Ghoneim,M.A., Stratton,M.R. and Venitt,S. (1995) Mutations in the *p53* gene in schistosomal bladder cancer: a study of 92 tumours from Egyptian patients and a comparison between mutational spectra from schistosomal and non-schistosomal urothelial tumours. *Carcinogenesis*, **16**, 1181–1189.
45. Maltzman,T.H., Mueller,B.A., Schroeder,J., Rutledge,J.C., Patterson,K., Preston-Martin,S. and Faustman,E.M. (1997) *Ras* oncogene mutations in childhood brain tumors [In Process Citation]. *Cancer Epidemiol. Biomarkers Prev.*, **6**, 239–243.
46. Hsieh,L.L., Hsieh,J.T., Wang,L.Y., Fang,C.Y., Chang,S.H. and Chen,T.C. (1996) *p53* mutations in gastric cancers from Taiwan. *Cancer Lett*., **100**, 107–113.
47. Liang,Y.Y., Esteve,A., Martel-Planche,G., Takahashi,S., Lu,S.H., Montesano,R. and Hollstein,M. (1995) *p53* mutations in esophageal tumors from high-incidence areas of China. *Int. J. Cancer*, **61**, 611–614.
48. Valavanis,C., Souliotis,V.L. and Kyrtopoulos,S.A. (1994) Differential effects of procarbazine and methylnitrosourea on the accumulation of O^6-methylguanine and the depletion and recovery of O^6-alkylguanine-DNA alkyltransfer*as*e in rat tissues. *Carcinogenesis*, **15**, 1681–1688.
49. Souliotis,V.L. and Kyrtopoulos,S.A. (1989) Development of a novel, sensitive assay for O^6-methyl and O^6-ethylguanine based on competitive repair by O^6-alkylguanine-DNA alkyltransferase. *Cancer Res*., **49**, 6997–7001.
50. Van Delft,J.H.M., Van Winden,M.J.M., Van den Ende,A.M.C. and Baan,R.A. (1993) Determining *N*7-alkylguanine adducts by immunochemical methods and HPLC with electrochemical detection — applications in animal studies and in monitoring human exposure to alkylating agents. *Environ. Hlth Perspect*., **99,** 25–32.
51. Mientjes,E.J., Steenwinkel,M.J., van Delft,J.H., Lohman,P.H. and Baan,R.A. (1996) Comparison of the X-gal- and P-gal-based systems for screening of mutant lambda *lac*Z phages originating from the transgenic mouse strain 40.6. *Mutat. Res*., **360**, 101–106.
52. Gossen,J.A., de Leeuw,W.J., Bakker,A.Q. and Vijg,J. (1993) DNA sequence analysis of spontaneous mutations at a *Lac*Z transgene integrated on the mouse X chromosome. *Mutagenesis*, **8**, 243–247.
53. Mientjes,E.J., Luiten-Schuite,A., van der Wolf,E., Borsboom,Y., Bergmans,A., Berends,F., Lohman,P.H.M., Baan,R.A. and van Delft,J.H.M. (1998) DNA adducts, mutant frequencies and mutation spectra in various organs of λ*lac*Z mice exposed to ethylating agents. *Environ. Mol. Mutagen*., **31**, 18–31.
54. Doolittle,D.J., Muller,G. and Scribner,H.E. (1987) A comparative study of hepatic DNA repair, DNA replication and hepatotoxicity in the CD-1 mouse following multiple administrations of dimethylnitrosamine. *Mutat. Res*., **188**, 141–147.
55. Doolittle,D.J., Muller,G. and Scribner,H.E. (1987) The *in vivo–in vitro* hepatocyte assay for assessing DNA repair and DNA replication: studies in the CD-1 mouse. *Food Chem. Toxicol*., **25**, 399–405.
56. Mirsalis,J.C., Tyson,C.K., Loh,E.N., Steinmetz,K.L., Bakke,J.P., Hamilton,C.M., Spak,D.K. and Spalding,J.W. (1985) Induction of hepatic cell proliferation and unscheduled DNA synthesis in mouse hepatocytes following *in vivo* treatment. *Carcinogenesis*, **6**, 1521–1524.
57. Lindamood,C., Bedell,M.A., Billings,K.C., Dyroff,M.C. and Swenberg,J.A. (1984) Dose-response for DNA alkylation, [^{3}H]thymidine untake into DNA

and O^6-methylguanine-DNA methyltransferase activity in hepatocytes of rats and mice continuously exposed to dimethylnitrosamine. *Cancer Res.*, **44**, 196–200.
58. Lee,V.M., Cameron,R.G. and Archer,M.C. (1993) The role of hepatocyte heterogeneity in the initiation of hepatocarcinogenesis. *Carcinogenesis*, **14**, 1403–1408.
59. Morrison,V. and Ashby,J. (1994) Reconciliation of five negative and four positive reports of the activity of dimethylnitrosamine in the mouse bone marrow micronucleus assay. *Mutagenesis*, **9**, 361–365.
60. Neal,S.B. and Probst,G.S. (1983) Chemically-induced sister-chromatid exchange *in vivo* in bone marrow of Chinese hamsters. An evaluation of 24 compounds. *Mutat. Res.*, **113**, 33–43.
61. Provost,G.S., Kretz,P.L., Hamner,R.T., Matthews,C.D., Rogers,B.J., Lundberg,K.S., Dycaico,M.J. and Short,J.M. (1993) Transgenic systems for *in vivo* mutation analysis. *Mutat. Res.*, **288**, 133–149.
62. Douglas,G.R., Gingerich,J.D., Gossen,J.A. and Bartlett,S.A. (1994) Sequence spectra of spontaneous *lac*Z gene mutations in transgenic mouse somatic and germline tissues. *Mutagenesis*, **9**, 451–458.
63. Jansen,J.G., de Groot,A.J., van Teijlingen,C.M., Tates,A.D., Vrieling,H. and van Zeeland,A.A. (1996) Induction of hprt gene mutations in splenic T-lymphocytes from the rat exposed *in vivo* to DNA methylating agents is correlated with formation of O^6- methylguanine in bone marrow and not in the spleen. *Carcinogenesis*, **17**, 2183–2191.
64. Akagi,T., Hiromatsu,K., Iyehara-Ogawa,H., Kimura,H. and Kato,T. (1993) Specificity of mutations induced by N-methyl-N-nitrosourea in a cDNA of the hprt gene. *Carcinogenesis*, **14**, 725–729.
65. Zhang,L.H. and Jenssen,D. (1991) Site specificity of N-methyl-N-nitrosourea-induced transition mutations in the hprt gene. *Carcinogenesis*, **12**, 1903–1909.
66. Horsfall,M.J. and Glickman,B.W. (1989) Mutational specificities of environmental carcinogens in the *lac*I gene of *Escherichia coli*. I. The direct-acting analogue N-nitroso-N-methyl-N-alpha-acetoxymethylamine. *Carcinogenesis*, **10**, 817–822.
67. Nivard,M.J., Pastink,A. and Vogel,E.W. (1996) Mutational spectra induced under distinct excision repair conditions by the 3 methylating agents N-methyl-N-nitrosourea, N-methyl-N′-nitro-N-nitrosoguanidine and N-nitrosodimethylamine in postmeiotic male germ cells of Drosophila. *Mutat. Res.*, **352**, 97–115.
68. Pletsa,V., Valavanis,C., van Delft,J.H.M., Steenwinkel,M.J.T. and Kyrtopoulos,S.A. (1997) DNA damage and mutagenesis induced by procarbazine in λ*lac*Z transgenic mice: Evidence that bone marrow mutations do not arise primarily through miscoding by O^6- methylguanine. *Carcinogenesis*, **18**, 2191–2196.
69. Voigt,J.M. and Topal,M.D. (1995) O^6-methylguanine-induced replication blocks. *Carcinogenesis*, **16**, 1775–1782.
70. Coquerelle,T., Dosch,J. and Kaina,B. (1995) Overexpression of N-methylpurine-DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents—a case of imbalanced DNA repair. *Mutat. Res.*, **336**, 9–17.
71. Ceccotti,S., Aquilina,G., Macpherson,P., Yamada,M., Karran,P. and Bignami,M. (1996) Processing of O^6-methylguanine by mismatch correction in human cell extracts. *Curr. Biol*, **6**, 1528–1531.
72. Ceccotti,S., Macpherson,P., Karran,P. and Bignami,M. (1994) O^6-methylguanine in DNA inhibits DNA replication and stimulates DNA repair synthesis *in vitro*. *Ann. NY Acad. Sci*., **726**, 340–342.
73. Guttenplan,J.B. (1993) Effects of cytosol on mutagenesis induced by N-nitrosodimethylamine, N-nitrosomethylurea and alpha-acetoxy-N-nitrosodimethylamine in different strains of Salmonella: evidence for different ultimate mutagens from N-nitrosodimethylamine. *Carcinogenesis*, **14**, 1013–1019.
74. Josephy,P.D., Lord,H.L. and Snieckus,V.A. (1994) Dimethylnitrosamine genotoxicity: does N-acetyltransferase activity play a role? *Carcinogenesis*, **15**, 479–482.
75. Shaham,J., Bomstein,Y., Meltzer,A., Kaufman,Z., Palma,E. and Ribak,J. (1996) DNA — Protein crosslinks, a biomarker of exposure to formaldehyde — *In vitro* and *in vivo* studies. *Carcinogenesis*, **17**, 121–125.
76. Ma,T.H. and Harris,M.M. (1988) Review of the genotoxicity of formaldehyde. *Mutat. Res.*, **196**, 37–59.
77. Liber,H.L., Benforado,K., Crosby,R.M., Simpson,D. and Skopek,T.R. (1989) Formaldehyde-induced and spontaneous alterations in human hprt DNA sequence and mRNA expression. *Mutat. Res.*, **226**, 31–37.
78. Crosby,R.M., Richardson,K.K., Craft,T.R., Benforado,K.B., Liber,H.L. and Skopek,T.R. (1988) Molecular analysis of formaldehyde-induced mutations in human lymphoblasts and *E.coli*. *Environ. Mol. Mutagen.*, **12**, 155–166.
79. Graves,R.J., Callander,R.D. and Green,T. (1994) The role of formaldehyde and S-chloromethylglutathione in the bacterial mutagenicity of methylene chloride. *Mutat. Res.*, **320**, 235–243.

Received on December 4, 1997; revised on February 16, 1998; accepted on February 16, 1998