Organ and Cell Specificity of DNA Methylation by N-Nitrosomethylamylamine in Rats

Michael Koenigsmann, Ivo Schmerold, Willy Jeltsch, Barbara Ludeke, Paul Kleihues, and Manfred Wiessler

ABSTRACT

N-Nitrosomethylamylamine (NMAA) is a potent carcinogen in rodents when administered chronically to BD IX rats s.c. or in the drinking water. Weekly i.p. injections in Wistar rats (2) revealed the additional induction of squamous cell papillomas and carcinomas of the trachea (up to 50% incidence) and the nasal cavity (up to 30%). As shown for several dialkynitrosamines (3), bioactivation of NMAA is initiated by enzymic hydroxylation, and this may occur at either the methyl group or at any of the carbon atoms of the 5-carbon chain. Following in vitro incubation of NMAA with rat liver microsomes, Mirvish et al. (4) were able to identify the intermediates hydroxylated at the C-2 to C-5 position of the amyl moiety. The α-carbon hydroxy intermediate, i.e., N-nitrosomethyl[1H-hydroxyamylamine, was not detectable, probably due to its short half-life. The authors concluded that the latter intermediate is most likely responsible for the adverse biological effects of NMAA since cleavage of the amyl moiety would produce methylidiazonium hydroxide as the ultimate carcinogen. This view is supported by a recent study from our laboratory on DNA methylation in various rat tissues by NMAA in vitro but until now no detailed report has been published on the extent of this reaction in a wide variety of target and non-target tissues in the intact rat.

We report the results of an investigation in which NMAA, 14C-labeled in the methyl group, was given as a single i.p. dose to male F344 rats, a strain previously used in systematic studies on the metabolism and reaction with DNA of asymmetrical aliphatic nitrosomethylalkylamines (5). In addition to radiochromatographic assessment of DNA methylation, autoradiographic and immunohistochemical studies were performed to localize cell-specific bioactivation within the known target tissues.

INTRODUCTION

Twenty years ago Druckrey et al. (1) reported that NMAA acts as a potent esophageal carcinogen when administered chronically to BD IX rats s.c. or in the drinking water. Weekly i.p. injections in Wistar rats (2) revealed the additional induction of squamous cell papillomas and carcinomas of the trachea (up to 50% incidence) and the nasal cavity (up to 30%).

As shown for several dialkynitrosamines (3), bioactivation of NMAA is initiated by enzymic hydroxylation, and this may occur at either the methyl group or at any of the carbon atoms of the 5-carbon chain. Following in vitro incubation of NMAA with rat liver microsomes, Mirvish et al. (4) were able to identify the intermediates hydroxylated at the C-2 to C-5 position of the amyl moiety. The α-carbon hydroxy intermediate, i.e., N-nitrosomethyl[1H-hydroxyamylamine, was not detectable, probably due to its short half-life. The authors concluded that the latter intermediate is most likely responsible for the adverse biological effects of NMAA since cleavage of the amyl moiety would produce methylidiazonium hydroxide as the ultimate carcinogen. This view is supported by a recent study from our laboratory on DNA methylation in various rat tissues by NMAA in vitro but until now no detailed report has been published on the extent of this reaction in a wide variety of target and non-target tissues in the intact rat.

We report the results of an investigation in which NMAA, 14C-labeled in the methyl group, was given as a single i.p. dose to male F344 rats, a strain previously used in systematic studies on the metabolism and reaction with DNA of asymmetrical aliphatic nitrosomethylalkylamines (5). In addition to radiochromatographic assessment of DNA methylation, autoradiographic and immunohistochemical studies were performed to localize cell-specific bioactivation within the known target tissues.

MATERIALS AND METHODS

Chemicals. [methyl-14C]NMAA was synthesized from K14CN (specific activity, 51 mCi/mmol; purchased from Hoechst AG, Frankfurt, Federal Republic of Germany) on a 500-μmol scale. K14CN, used without further dilution, was hydrogenated (platinum/carbon, H2/O2/CH3OH) to [14C]methylenamin HCl. Reaction with valeranaldehyde in neutral aqueous solution yielded a Schiff’s base which gave [14C]-methylenamin HCl upon hydrogenation (platinum/carbon, aqueous ethanol). Nitrosoation in aqueous solution with NaN3 produced [methyl-14C]NMAA with a yield of 20% (based on K14CN). The specific activity was 58.3 mCi/mmol. The radiochemical purity was checked by high pressure liquid chromatography on RP-18 columns (Shandon ODS Hypersil, 4.6 × 250 mm, eluted with 40% aqueous methanol and UV detection at 230 nm) and was found to be 98%. Before use, unlabeled N-nitrosomethylamylamine (obtained from Dr. W. Lijinsky, National Cancer Institut-Frederick Cancer Research Facility, Frederick, MD), was added to decrease the specific activity to 9.14 mCi/mmol. Sephasorb-HP was purchased from Pharmacia, Uppsala, Sweden. Lumagel SB was from Lumac/3M, Schaesberg, The Netherlands. Hydroxylapatite (DNA grade) was from Bio-Rad Laboratories AG, Glatzbrugg, Switzerland. RNase T1, from Aspergillus oryzae was obtained from Boehringer-Mannheim AG, Rotkreuz, Switzerland, RNase A from bovine pancreas, ovalbumin and 3,3-diaminobenzidine-4HCl were purchased from Sigma Chemie, D-8024 Deisenhofen, Federal Republic of Germany. Peroxidase-rabbit antiperoxidase complex, swine anti-rabbit immunoglobulin (raised against rabbit serum pool), and nonimmune swine serum were from Dakopatts AS, Glostrup, Denmark. All other chemicals were of analytical grade or higher.

Animal Treatment. Male Fischer 344 rats weighing 110–150 g (Charles River Wiga, Federal Republic of Germany) were maintained on a commercial diet with water ad libitum. For the determination of methylated DNA bases, 8 rats were given a single i.p. injection of [methyl-14C]NMAA. To make this experiment compatible with earlier fluorescence studies (5), we chose a dose of 0.1 mmol/kg, corresponding to 13 mg/kg body weight. After a survival time of 6 h, the animals were killed by exsanguination during ether anesthesia. Organs (liver, kidney, spleen, stomach, duodenum, lungs, esophagus, trachea) were rapidly removed, frozen in liquid N2, and stored at −70°C. Material from the nasal cavity was obtained by curettage after median splitting of the...
head and was processed in the same way as the other tissues. For autoradiographic studies, two rats were pretreated with an i.p. injection of hydroxyurea (500 mg/kg) followed by an i.p. dose of [methyl-14C]-NMAA (9.14 mCi/mmol, 0.1 mmol/kg) 30 min later. Animals were sacrificed after an additional survival time of 2 h. For the immunohistochemical identification of cells containing O\(^\text{6}\)-methylenoxyguanosine, 3 rats received a single i.p. injection of 1.3, 13, or 65 mg NMAA/kg body weight, corresponding to 0.01, 0.1, and 0.5 mmol/kg (survival time, 4.5 h).

Radiochromatography. DNA was isolated by adsorption onto hydroxyapatite using a modification (7) of the methods reported by Viviani and Lutz (8) and Markov and Ivanov (9). Following hydrolysis in 0.1 M HCl at 37°C for 20 h DNA from the pooled organs of 8 rats (1–2 g) was analyzed by Sephasorb chromatography (10). Guanine and adenine were determined by their absorption at 260 nm and 14C-labeled purines were quantified by liquid scintillation counting (counting efficiency, 85%). The concentrations of methylated purines were calculated assuming that their specific radioactivity was identical to that of the nitrosamine injected.

 Autoradiography. Tissues were fixed in buffered formaldehyde (4%, v/v), embedded in paraffin, and cut into 4-μm sections. Exposure of contact autoradiographs (LKB 3H-Utrofilm) was carried out for 3 weeks in an X-ray cassette. Films were processed with Kodak D19 developer and Kodak Unifix fixation salt.

 Immunohistochemistry. The organs were removed rapidly and quickly frozen onto small aluminum plates placed directly on slabs of dry ice. Characteristics of the rabbit antiserum raised against keyhole limpet hemocyanin conjugates of O\(^\text{6}\)-methylguanosine (NPZ 193-1) have been described earlier (11). Briefly, we found 3-fold lower reactivity with O\(^\text{6}\)-methyldeoxyguanosine and no cross-reactivity with O\(^\text{6}\)-hydroxyethyldeoxyguanosine. Neither adduct has been shown to result from the reaction of NMAA metabolites with cellular DNA. The antiserum was used without prior absorption. The procedure of Heyting et al. (12) and Menkveld et al. (13) was used with several modifications, generously communicated by Dr. E. Scherer and colleagues at The Netherlands Cancer Institute, Amsterdam, The Netherlands. Cryostat sections (6–10 μm) were mounted on ovalbumin-coated slides. Endogenous peroxidase was inactivated by a 45-min incubation with 0.3% H\(_2\)O\(_2\) in methanol (14). After rehydration via graded ethanol, sections were equilibrated with 10 mM EDTA and 10 mM Tris, pH 8.0, for 5 min and subsequently treated for 60 min at 37°C with RNase A (200 μg/ml) and RNase T\(_1\) (50 units/ml) in the same buffer. The sections were then rinsed with distilled water and fixed for 1 min with 40% ethanol, treated for 10 min at room temperature with 50 mM NaOH in 40% ethanol to denature the DNA, neutralized with 5% glacial acetic acid in 40% ethanol, rinsed once with water, incubated for 5 min in wash buffer (50 mM Tris, pH 7.4–150 mM NaCl–5 mM EDTA-0.25% gelatin–0.05% Triton X-100), and then rinsed in PBS. The sections were subsequently preincubated (60 min, 37°C) with antibody dilution buffer (10% heat-inactivated nonimmune swine serum in PBS containing 0.04% Triton X-100). The reaction with the anti-O\(^\text{6}\)-methylenoxyguanosine serum (diluted 1:5,000 or 1:10,000; see figure legends) was carried out for 16 h at 4°C. After this and all following incubation steps, the sections were washed once with PBS, once with wash buffer, and again with PBS. Bound antibodies were detected by the "double peroxidase-antiperoxidase" staining procedure (15), which involved successive incubations with swine anti-rabbit immunoglobulin, peroxidase-(rabbit)antiperoxidase complex, swine anti-rabbit immunoglobulin, and peroxidase-(rabbit)antiperoxidase complex, each carried out for 45 min at room temperature. Enzymatic activity was visualized by incubation in 50 mM Tris-HCl (pH 7.4), 3,3-diaminobenzidine-4HCl (0.5 mg/ml), and 0.015% H\(_2\)O\(_2\) for 5–10 min at room temperature. Before further processing (dehydration and mounting), the sections were washed with distilled water.

**RESULTS**

Following a single i.p. administration of [methyl-14C]-NMAA at a dose of 13 mg/kg, DNA methylation was detectable in all tissues investigated (Table 1). Due to the small volume of some of the tissues (e.g., esophagus, trachea, nasal epithelium), DNA analyses had to be performed on pooled tissue samples from 8 animals. To make the results overall compatible, the same procedure was applied to all other tissues. However, the radiochromatographic determination of alkylpurines is very precise and triplicate determinations on pooled liver samples differed from each other by less than 4%, the standard deviation amounting to less than 2% of the mean. Concentrations of 7-methylguanine were highest in esophagus, nasal cavity, and liver, followed by trachea and lung. Foremost stomach and kidney values were approximately 30–40 times lower than in esophagus. In the glandular stomach, spleen, and duodenum, the amount of 7-methylguanine was close to the limit of detection.

**Table 1 DNA methylation by N-nitrosomethylamylamine in vivo**

<table>
<thead>
<tr>
<th>Organ</th>
<th>7-Methylguanine</th>
<th>O(^\text{6})-Methylguanine</th>
<th>7-Methylguanine: O(^\text{6})-Methylguanine ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus</td>
<td>798</td>
<td>91</td>
<td>0.11</td>
</tr>
<tr>
<td>Nasal epithelium</td>
<td>672</td>
<td>79</td>
<td>0.12</td>
</tr>
<tr>
<td>Liver</td>
<td>624</td>
<td>50</td>
<td>0.08</td>
</tr>
<tr>
<td>Trachea</td>
<td>214</td>
<td>26</td>
<td>0.12</td>
</tr>
<tr>
<td>Lung</td>
<td>101</td>
<td>10</td>
<td>0.10</td>
</tr>
<tr>
<td>Foremostomach</td>
<td>25</td>
<td>±1</td>
<td>ND*</td>
</tr>
<tr>
<td>Kidney</td>
<td>20</td>
<td>±1</td>
<td>ND*</td>
</tr>
<tr>
<td>Glandular stomach</td>
<td>4</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>ND</td>
<td>ND*</td>
</tr>
<tr>
<td>Duodenum</td>
<td>2</td>
<td>ND</td>
<td>ND*</td>
</tr>
</tbody>
</table>

* Pooled tissues from 8 animals.

# ND, not detectable.

Fig. 1. H & E stain (top) and histoautoradiograph (bottom) of transverse sections through the upper thorax of a male F344 rat following a single i.p. dose of N-nitrosomethylamylamine (0.1 mmol/kg body weight; survival time, 2 h). In order to suppress DNA synthesis, the animal was given a single i.p. injection of hydroxyurea 30 min before nitrosamine application. E, esophagus; T, trachea; L, lung; H, heart. × 6.
Fig. 2. H & E stain (A) and histoautoradiograph (B) of frontal sections through nose and palate of an F344 rat. For animal treatment see legend to Fig. 1. E, ectoturbinalia; N, endoturbinalia; P, palatal mucosa; O, olfactory epithelium; R, respiratory epithelium; S, Steno's (lateral nasal) glands. x 8.

Fig. 3. Immunocytochemical localization of O6-methyldeoxyguanosine in the esophageal mucosa (A), olfactory (B), and bronchial (C) and tracheal (D) epithelium of a male F344 rat 4.5 h following a single i.p. injection of N-nitrosomethylamylamine (0.5 mmol/kg). The rabbit antiserum was used at dilutions of 1:10,000 (A) and 1:5,000 (B, C, D). A, x 120; B and C, x 240; D, x 480.

Quantifiable amounts of O6-methylguanine were present only in esophagus, nasal cavity, liver, trachea, and lung. In these tissues the O6-methylguanine/7-methylguanine ratio was close to 0.11 except in liver (0.08). In order to study the site of NMAA bioactivation at the cellular level, histoautoradiographic examinations were carried out in rats that had received a similar dose of [methyl-14C]NMAA. On a cross-section through tissues of the upper thoracic cavity including the mediastinum (Fig. 1), incorporation of 14C into macromolecules was most accentuated in the esophageal mucosa. Other target
sites easily identifiable were the trachea and bronchial tree. The remaining lung parenchyma showed diffuse and considerably less marked incorporation of radioactivity. In the nasal cavity, intensive radiolabeling was present both in the olfactory and respiratory epithelium and in the lateral nasal glands (Steno’s glands (Fig. 2)). Unexpectedly, we also observed a considerable amount of radioactivity incorporated into the palatal mucosa. The liver showed an inhomogeneous distribution of radioactivity with predominant labeling of the centrilobular areas. In the kidney, incorporation of $^{14}$C was generally less extensive, with predominant labeling of the inner cortical layers whereas the medullary zone showed only very faint binding (not shown). The intraorgan distribution of reaction products as revealed by autoradiography was largely confirmed by immunohistochemical studies using a rabbit antiserum to $O^6$-methyldeoxyguanosine. In the esophagus, this promutagenic base was only detectable in the mucosal epithelium, in particular in the stratum basale and in nuclei of cells with incipient keratinization (Fig. 3A). Similarly, peroxidase-labeled nuclei were present in the olfactory and respiratory epithelium of the nasal cavity including the ductal epithelia of Bowman’s glands (Fig. 3B). More diffuse staining for $O^6$-methyldeoxyguanosine was observed in the lateral nasal glands (not shown). In the lower respiratory tract, the immunohistochemical detection of $O^6$-methyldeoxyguanosine was largely restricted to the tracheal mucosa (Fig. 3D) and the bronchiolar tree. Within the bronchiolar epithelium, staining was somewhat inhomogeneous (Fig. 3C) but no attempt was made to identify the most heavily labeled nuclei as those of Clara cells. No immunoreactivity was detected when incubations were carried out with normal rabbit serum or with tissues from untreated control animals.

**DISCUSSION**

The systematic investigations by Druckrey et al. (1) revealed that the most powerful esophageal carcinogens in rats are asymmetrical nitrosoamines with a methyl group as one of the alkyl moieties. Prominent examples of this class of carcinogens are $N$-nitrosomethylbenzylamine, $N$-nitrosomethylcyclohexylamine and NMAA. For the homologous series of aliphatic nitrosomethylalkylamines it has been shown that those with a chain length of 3-6 carbon atoms are most effective (5). Initiation of malignant transformation in the esophageal mucosa by these compounds is largely independent of the route of administration, indicating that the initial distribution of the parent carcinogen is not a key factor in organ specificity. This is supported by pharmacokinetic studies which in the case of $N$-nitrosomethylbenzylamine (2.5 mg/kg) and NMAA (25 mg/kg) showed clearance from rat serum at an apparent half-life of 35 and 21 minutes, respectively (6, 16). Preferential bioactivation of these esophageal carcinogens in the target tissue, i.e., the esophageal mucosa, was first shown for $N$-nitrosomethylbenzylamine which, after systemic administration, was found to methylate esophageal DNA 3-4 times more extensively than that of rat liver (16). In the present study with NMAA, DNA methylation was also highest in the esophagus followed by nasal epithelium, liver, trachea, and lung. The esophagus: liver ratio (1.28) is somewhat higher than that observed by high performance liquid chromatography-fluorescence detection in DNA (0.98) of rats exposed to a similar dose administered by stomach tube (5). In most tissues, the $O^6$-methylguanine ratio was 0.11 or very close to this value which is typically observed after reaction of methylating $N$-nitroso compounds with DNA in vitro, indicating that during the 6 h observation period, $O^6$-methylguanine was not repaired to any significant extent. The lower ratio of 0.078 in hepatic DNA reflects the high activity of the repair enzyme, DNA-$O^6$-methylguanine:protein-$L$-cysteine $S$-methyltransferase in rat liver.

The present autoradiographic and immunohistochemical studies clearly show that bioactivation of NMAA in the esophagus occurs in the mucosa proper (Fig. 1), with highest levels of reaction in the stratum basale (Fig. 3A). A similar distribution of $O^6$-ethyldeoxyguanosine within the esophageal mucosa was recently observed by Scherer et al. (17) in rats treated with a single dose of $N$-nitrosodiethylamine. Within the respiratory tract, NMAA was found to be bioactivated in the nasal cavity (respiratory and olfactory epithelium, Steno’s and Bowman’s glands) and in the epithelia of the trachea and of the entire bronchial tree. Autoradiography (Fig. 1) also revealed diffuse incorporation of $^{14}$C into the remaining lung parenchyma but immunohistochemical staining of $O^6$-methyldeoxyguanosine appeared to be restricted to the bronchiolar epithelium (Fig. 3C). Although both methods were found to yield similar results, the immunohistochemical technique developed by Menkveld et al. (13) must be considered superior. Autoradiography with dehydrated, paraffin-embedded sections visualizes $^{14}$C radioactivity bound to cellular macromolecules, including DNA, RNA, and proteins. However, labeling may to a considerable extent be due to metabolic incorporation via the C-1 pool, particularly in tissues with a high cell turnover, e.g., spleen, bone marrow, and intestines. We have attempted to minimize metabolic incorporation by a short survival time (2 h) and pretreatment with hydroxyurea which at a dose of 500 mg/kg causes almost complete inhibition of DNA synthesis over a period of three h (18). Radiochromatographic analyses revealed that in rat duodenum, a replicating tissue notorious for high metabolic incorporation, pretreatment with hydroxyurea led to a reduction of purine labeling by more than 98% (data not shown). Nevertheless, $^{14}$C labeling of the gastric mucosa (fundus) was eminent on autoradiographs although biochemical analyses had shown that the extent of DNA methylation in this tissue is close to the level of detection (Table 1). In contrast, immunohistochemical detection of modified bases, although less sensitive, is carried out with antibodies specific for a chemically defined adduct, e.g., $O^6$-methyldeoxyguanosine. Since slides are incubated with RNase A and T1 during processing to eliminate cross-reactivity with RNA bases, the presence of a specific DNA modification in cell nuclei is revealed.

If the extent of DNA methylation were the only determining factor in the initiation of malignant transformation by NMAA, one would expect a similar tumor incidence in the esophagus, nasal cavity, and tracheobronchial system. However, esophageal neoplasms of the rat including precancerous papillomas develop after a very short latency period and, through impaired food intake, often lead to the animal’s death within 2 to 3 months after the onset of nitrosamine administration. Tracheal and nasal carcinomas are, therefore, more frequently encountered in animals with a longer survival period (2). From the autoradiograph shown in Fig. 2, we would anticipate that the mucosa of the oral cavity is also prone to tumor induction by NMAA. It has indeed been observed that after administration in the drinking water together with catechol, NMAA occasionally induces tumor of the tongue.*

The organ-specific pattern of DNA alkylation observed in the present study strongly suggests the presence of a $P$-450 isoyme with a high substrate specificity for NMAA and related

* S. Mirvish, Ep plyle Institute for Cancer Research, Omaha, NB, personal communication.
asymmetrical nitrosamines in epithelia derived from the ventral entoderm, i.e., the upper respiratory and gastrointestinal tract. Anatomically, the most distal level at which this enzyme is located appears to be the border between esophagus and stomach. Although the latter organ contains a squamous epithelium histologically indistinguishable from that of the esophagus, it shows 30-fold lower levels of DNA methylation by both NMAA and N-nitrosomethylbenzylamine (16). The substrate specificity of this enzyme system, however, may not be restricted to methylaryl- and aliphatic methylalkylnitrosamines. Extensive DNA methylation in the nasal cavity and the bronchiolar mucosa was methylated to a similar extent whereas at doses below 10 mg/kg/day preferential DNA methylation was observed in Clara cells, which have been identified as possible progenitors for nitrosamine-induced lung tumors in rodents (24, 25). It remains to be shown whether similar cell-specific low Km pathways exist for the pulmonary and nasal bioactivation of NMAA, N-nitrosomethylbenzylamine, and related asymmetrical nitrosamines.

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