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DNA methylation in the digestive tract of F344 rats during chronic exposure to *N*-methyl-*N*-nitrosourea

Hiroko Ohgaki [1,2], Barbara I. Ludeke [2], Irene Meier [1], Paul Kleihues [2], Werner K. Lutz [1], and Christian Schlatter [1]

[1] Institute of Toxicology, Federal Institute of Technology and University of Zürich, CH-8603 Schwerzenbach, Switzerland
[2] Laboratory of Neuropathology, Institute of Pathology, University of Zürich, CH-8091 Zürich, Switzerland

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Summary. The formation of O^6-methyldeoxyguanosine (O^6-MedGuo) was determined by an immuno-slot-blot assay in DNA of various tissues of F344 rats exposed to *N*-methyl-*N*-nitrosourea (MNU) in the drinking water at 400 ppm for 2 weeks. Although the pyloric region of the glandular stomach is a target organ under these experimental conditions, the extent of DNA methylation was highest in the forestomach (185 μmol O^6-MedGuo/mol guanine). Fundus (91 μmol/mol guanine) and pylorus (105 μmol/mol guanine) of the glandular stomach, oesophagus (124 μmol/mol guanine) and duodenum (109 μmol/mol guanine) showed lower levels of O^6-MedGuo but differed little between each other. Thus, no correlation was observed between target organ specificity and the extent of DNA methylation. This is in contrast to the gastric carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), which preferentially alkylates DNA of the pylorus, the main site of induction of gastric carcinomas by this chemical. In contrast to MNU, the non-enzymic decomposition of MNNG is accelerated by thiol compounds (reduced glutathione, L-cysteine), which are present at much higher concentrations in the glandular stomach than in the forestomach and oesophagus. During chronic exposure to MNNG (80 ppm), mucosal cells immunoreactive to O^6-MedGuo are limited to the luminal surface [Kobori et al. (1988) Carcinogenesis 9:2271–2274]. Although MNU (400 ppm) produced similar levels of O^6-MedGuo in the pylorus, no cells containing methylpurines were detectable by immunohistochemistry, suggesting a more uniform methylation of mucosal cells by MNU than by MNNG. After a single oral dose of MNU (90 mg/kg) cells containing methylpurines were unequivocally identified using antibodies to O^6-MedGuo and the imidazole-ring-opened product of 7-methyldeoxyguanosine. In the gastric fundus, their distribution was similar to those methylated by exposure to MNNG, whereas the pyloric region contained immunoreactive cells also in the deeper mucosal layers. After a 2-week MNU treatment, the rate of cell proliferation, as determined by bromodeoxyuridine immunoreactivity, was only slightly enhanced in the oesophagus and in the fundus, but markedly in the forestomach and the pyloric region of the glandular stomach. It is concluded that the overall extent of DNA methylation, the distribution of alkylated cells within the mucosa and the proliferative response all contribute to the organ-specific carcinogenicity of MNU.

Key words: Gastric carcinogenesis – *N*-methyl-*N*-nitrosourea – DNA methylation

Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; O^6-MedGuo, O^6-methyldeoxyguanosine; 7-MedGuo, 7-methyldeoxyguanosine (imidazole ring open); BrdUrd, bromodeoxyuridine

Offprint requests to: H. Ohgaki, Abteilung Neuropathologie, Institut für Pathologie, Universitätsspital, CH-8091 Zürich, Switzerland

Introduction

The target organ specificity of *N*-methyl-*N*-nitrosourea (MNU) largely depends on the route of administration. A single systemic (i.v.) dose induces neoplasms in various tissues (Druckrey et al. 1967; Murthy et al. 1973) whereas chronic i.v. administration leads to the preferential induction of brain tumours in rats (Druckrey et al. 1964, 1965). It has been reported that chronic administration of MNU in the drinking water induced forestomach tumours in BD and Donryu rats (Druckrey et al. 1961; Ogiu et al. 1977). More recently, it has been shown that MNU also induces a high incidence of carcinomas in the pyloric region of the glandular stomach when given to F344 rats continuously in the drinking water (Maekawa et al. 1985; Hirota et al. 1987; Fujita et al. 1989). The site-specific induction of gastric carcinomas by MNU is a somewhat surprising observation since MNU acts as a methylating agent after non-enzymatic hydrolysis and chemical rearrangements to the methyldiazonium ion. Its relative stability in vivo allows for homogeneous systemic distribution (Kleihues and Patzschke 1971), with similar

levels of DNA methylation in different tissues (Kleihues and Magee 1973). In contrast, the decomposition of the gastric carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is enhanced by thiols (Lawley and Shah 1972; Wheeler and Bowdon 1972) which have been shown to occcur at higher concentrations in the glandular stomach than in the more proximal segments of the gastrointestinal tract (Boyd et al. 1979; Wiestler et al. 1983). Furthermore, MNU is, unlike MNNG, stable under acidic conditions in gastric juice (Druckrey et al. 1967; McKay and Wright 1947). Thus, the mechanisms of gastric tumour induction by MNU and MNNG appear to be different.

The target organ specifity for genotoxic carcinogens is based primarily on the level of DNA damage in the proliferative cells. The aim of the present study was, therefore, to investigate DNA methylation (concentration and distribution), the rate of cell proliferation, and the hyperplastic response in various parts of the gastrointestinal tract of the rats during chronic administration of MNU in the drinking water. An immuno-slot-blot assay (Nehls et al. 1984; Ludeke and Kleihues 1988) was used to determine the concentration of O^6-methyldeoxyguanosine (O^6-MedGuo). In addition, cells containing methylpurines were identified immunohistochemically using antibodies to O^6-MedGuo and the imidazole ring-opened form of 7-methyldeoxyguanosine (7-MedGuo). Proliferating cells were identified by immunocytochemistry for bromodeoxyuridine (BrdUrd).

Materials and methods

Carcinogens. MNU (stabilized with acetic acid) and MNNG were purchased from Sigma (St. Louis, USA) and Fluka Chemie AG (Buchs, Switzerland), respectively. Tenfold stock solutions of MNU were prepared in distilled water (4000 ppm) and kept at 4° C under light protection. For animal studies, it was diluted with distilled water immediately before use. Drinking solutions were changed daily except on weekends. The stability of the MNU solution before and after use was determined by measuring the absorbance at 231 nm on a Hewlett Packard spectrophotometer.

Animal experiments. Young male F344 rats (100–150 g body weight) were purchased from Savo GmbH Kisslegg (FRG), housed four to a cage in macrolone cages and maintained on a basal diet (NAFAG 890.1664, NAFAG, Gossau, Switzerland). A group of 20 rats were given MNU in the drinking water (400 ppm) ad libitum. After treatment for 2 weeks, rats were sacrified and brain, oesophagus, duodenum, colon, liver, and stomach (forestomach, pylorus and fundus of the glandular stomach) were removed. Organs from 10 rats were frozen separately in liquid nitrogen and kept at −80° C. For immunohistochemical studies, organs from 5 rats were frozen on solid CO_2 and kept at −80° C until staining. Five rats were injected i.p. with BrdUrd (20 mg/kg) 1 h before sacrifice and organs were fixed in 70% ethanol (v/v) for 2 h. Two control rats were similarly injected with BrdUrd. Two rats were treated with a single dose of MNU (1.5 ml, 90 mg/kg body weight; 50% of LD_{50}) or MNNG solution (2.0 ml 83 ppm solution; 1.3 mg/kg body weight) by instillation in the upper oesophagus. After 2 h, rats were killed and organs were removed as described above for immunohistochemistry.

Body weight, water consumption and carcinogen intake. The mean body weights of rats chronically exposed to MNU decreased about 15% during the first week of treatment but remained stable the second week of treatment. The water consumption per rat was, on the average, 7.6 ml/day. During the first week, the mean concentration of the MNU solution (pH 4.5), as calculated from the UV absorbance before and after application, was 320 ppm. Since the body weight decreased during the first week, the pH of the drinking solution was adjusted by NaOH to 6–7 during the second week, which resulted in a more rapid decay of MNU. Under these conditions the calculated mean concentration of the MNU solution was 130 ppm. The daily MNU intake, calculated from water consumption and MNU concentrations, was 11.9 mg/kg body weight/day.

Immuno-slot-blot assay. The characteristics of the rabbit antiserum to O^6-MedGuo (NPZ193-1) and of the alkylated DNA standards have been described previously (Ludeke and Kleihues 1988). DNA was purified by phenol extraction using a nucleic acid extractor (model 340A; Applied Biosystems, Foster City, Calif. 94404, USA). Since the amounts of DNA obtained from some tissues (oesophagus, forestomach, pylorus) were too small to allow for individual analysis, the organs from two groups of 5 animals each were pooled before DNA isolation (Table 1). To test the individual differences in the levels of O^6-MedGuo, triplicate analyses were carried out on fundus and liver of rats given MNU for 2 weeks. Inter-individual values for O^6-MedGuo differed by less than 30% and the standard deviation was less than 20% of the mean.

Details of the immuno-slot-blot assay were described earlier (Ludeke and Kleihues 1988). Briefly, DNA samples (9 µg in 300 µl) were heat-denatured for 10 min in boiling water, quickly chilled on ice, and mixed with an equal volume of 2 *M* ammonium acetate. DNA was immobilized on nitrocellulose filters (BA 85, Schleicher and Schüll). Filters were presoaked in 1 *M* ammonium acetate and 3 µg DNA in 200 µl was applied to each well. After rinsing each well with 200 µl 1 *M* ammonium acetate, filters were removed from the support. The filters were soaked in 0.75 *M* NaCl, 0.075 *M* trisodium citrate for 5 min, blow-dried and baked at 80° C for 2.5 h. After blocking non-specific protein-binding sites with 0.5% casein in phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 m*M* KCl, 8.1 mM Na_2HPO_4 $2H_2O$, and 1.5 m*M* KH_2PO_4, pH 7.2) at 37° C for 30 min, the filters were incubated overnight at 4° C with anti-O^6MedGuo antibody diluted 1:8000 in the same buffer. The filters were subsequently washed three times in casein buffer, once in high-saline PBS (PBS-HS), and once in PBS-HS containing 0.05% Nonidet P-40 (Sigma), allowing 10 min for each buffer change. Then, filters were incubated with goat anti-(rabbit IgG)–alkaline-phosphatase conjugate (Bio-Rad, Richmond, Calif.), diluted 1:3000 in PBS containing 0.5% casein for 3 h at room temperature. The nitrocellulose filters were then washed as above. Enzymatic activity was visualized by incubation for 10–20 min in a solution of 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Bio-Rad, Richmond, Calif.) and *p*-nitroblue tetrazolium chloride (Bio-Rad, Richmond, Calif.) in diethanolamine buffer (0.1 *M* NaCl, 0.1 *M* TRIS, 25.0 m*M* diethanolamine, 2.0 m*M* $MgCl_2$, and 1.0 µ*M* $ZnCl_2$, pH 9.55) at 37° C according to Ey and Ashman (1986). The filters were subsequently rinsed extensively with distilled water and stored wet in the dark until densitometry. Densitometric evaluation was performed using a Shimadzu model CS-930 dual-wavelength thin-layer chromatogram scanner in the zig-zag mode at 530 nm.

Immunohistochemistry for alkylated DNA bases. The polyclonal antibody to O^6-MedGuo was the same as that used in the immuno-slot-blot assay. A monoclonal antibody to 7-MedGuo (Montesano et al. 1988) was kindly provided by Dr. Christopher Wild, International Agency for Research on Cancer, Lyon, France. The immunohistochemical reaction used (Koenigsmann et al. 1988) was a modification of the procedure described by Menkveld et al. (1985). Briefly, cryostat sections (6–10 µm) were mounted on slides that were coated with 3-aminopropylmethoxysilane. Endogenous peroxidase was inactivated by a 45-min incubation with 0.3% H_2O_2 in methanol. After dehydration, sections were incubated with 10 m*M* EDTA and 10 m*M* TRIS, pH 8.0, for 5 min and subsequently treated with RNase A (200 µg/ml; Sigma, St. Louis, USA) and RNase T_1 (50 U/ml; Boehringer Mannheim, FRG) in the same buffer for 60 min at 37° C. Sections were rinsed with distilled water and fixed with 40% ethanol for 1 min, then treated with 50 m*M* NaOH for 10 min in 40% ethanol to denature DNA. After neu-

tralizing with 5% glacial acetic acid in 40% ethanol and washing with water, sections were incubated for 5 min in wash buffer (50 m*M* TRIS, pH 7.4, 150 m*M* NaCl, 5 m*M* EDTA, 0.25% gelatine), and rinsed with PBS. The sections were subsequently pre-incubated (60 min, 37° C) with antibody-dilution buffer (10% heat-inactivated non-immune swine serum in PBS). The reaction with the anti-O^6-MedGuo or anti-7-MedGuo serum (diluted 1:10000 or 1 5000, respectively) was carried out for 16 h at 4° C. Bound antibodies were detected by the double PAP procedure (Ordronneau et al. 1981), which involved successive incubations with swine anti-(rabbit Ig), peroxidase-(rabbit)antiperoxidase complex, swine anti-(rabbit Ig) and peroxidase-(rabbit)antiperoxidase complex, each carried out for 30 min at room temperature. Enzymatic activity was visualised by incubation in 0.035% 3,3-diaminobenzidine·4HCl in 50 m*M* TRIS/HCl, pH 7.4, containing 0.015% H_2O_2 for 5–10 min at room temperature.

BrdUrd immunohistochemistry. The immunohistochemical technique was essentially that described previously (Ohgaki et al. 1988). Briefly, paraffin sections were deparaffinized in xylene and placed in absolute ethanol. Endogenous peroxidase activity was blocked by incubating them for 30 min in 0.3% H_2O_2 in methanol. The sections were washed with distilled water and then incubated in 2 M HCl for 30 min. Neutralization was done by incubation in 0.1 *M* $Na_2B_4O_7$ for 10 min. The sections were treated with 10% normal swine serum and then incubated with anti-BrdUrd monoclonal antibody (diluted 1:20; Becton Dickinson, Basel, Switzerland) for 2 h at room temperature. After this and following the incubation steps, the sections were washed three times with PBS (pH 7.4). The sections were incubated with biotinylated anti-(mouse-IgG) (diluted 1:100; Amersham, UK) for 30 min and then avidin-biotin-peroxidase complex (DAKO, Denmark) for 30 min. Sections were developed in 0.05% 3,3-diaminobenzidine·4HCl in 50 m*M* TRIS/HCl buffer (pH 7.4) containing 0.03% H_2O_2 for 5–10 min at room temperature. The sections were stained lightly with haematoxylin and examined histologically.

Results

Histopathology and cell proliferation after 2 weeks of exposure to MNU

After treatment with MNU for 2 weeks (400 ppm in the drinking water), the oesophageal epithelium was slightly, and that of the forestomach severely hyperplastic, with concomitant hyperkeratosis. Cell proliferation in the oesophagus was slightly enhanced whereas that in the forestomach was prominently increased when compared to untreated control rats. No histological changes were found in the fundic region of the glandular stomach, but in the pyloric region, erosion, elongation of pits, and irregular arrangement of glands were present in all rats given MNU for 2 weeks. A slight increase in the fraction of BrdUrd-labelled cells was observed in the fundic region but this was much more pronounced in the pylorus. In the duodenum, a slight elongation of villi was found, together with a slight increase in the number of proliferating cells. No histological changes were detected in colon, liver or brain.

Formation of O^6-MedGuo

Significant amounts of O^6-MedGuo were found in all organs of rats that were given MNU for 2 weeks (Table 1). DNA methylation was highest in the brain. Within the gastrointestinal tract, forestomach showed highest levels of methylation. Concentrations of O^6-MedGuo in oesophagus, pylorus, fundus, and duodenum were lower but differed little from each other. Colon and liver showed the lowest amounts of O^6-MedGuo.

Table 1. Organ-specific levels of O^6-MedGuo in DNA in rats given *N*-methyl-*N*-nitrosourea (MNU) in the drinking water (400 ppm) for 2 weeks[a]

Organ	O^6-MedGuo (mol/mol guanine)		
	Group 1	Group 2	Mean
Oesophagus	114	134	124
Forestomach	197	172	185
Glandular stomach			
Fundus	87	94	91
Pylorus	97	112	105
Duodenum	103	116	109
Colon	62	55	59
Liver	36	23	29
Brain	230	226	228

[a] The organs from two groups of 5 animals each were pooled before DNA isolation. To test the individual differences in the levels of O^6-MedGuo, triplicate analyses were carried out on fundus and liver of rats given MNU for 2 weeks. Inter-individual values for O^6-MedGuo differed by less than 30% and the standard deviation was less than 20% of the mean

Immunohistochemical identification of cells containing methylpurines

After 2 weeks of exposure to MNU in the drinking water, no immunoreactivity was detectable in any of the tissues examined. In contrast, in rats given a single high dose of MNU (90 mg/kg body weight), surface epithelia of the fundus and pylorus of the glandular stomach were stained intensely by the anti-O^6-MedGuo and anti-7-MedGuo antibodies. In gastric fundus, the population of cells stained with anti-O^6-MedGuo and anti-7-MedGuo antibodies was limited to the luminal surface (Fig. 1a) whereas in the pylorus, deeper mucosal layers also showed unequivocal immunoreactivity (Fig. 1b). There were no positive cells in the other organs of rats given a single dose of MNU. For comparison, two rats were given a single dose of MNNG (1.3 mg/kg) by instillation in the upper oesophagus. Cells staining for O^6-MedGuo and 7-MedGuo were observed in the surface epithelium of both the fundic and pyloric region of the glandular stomach (Fig. 1c, d).

Discussion

Induction of gastric carcinomas by MNNG and its ethyl analogue, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine constitutes the major animal model that has been studied extensively in various laboratories (Ohgaki and Sugimura 1988). In rats, chronic exposure to MNNG in the drinking water induces a high incidence of carcinomas in the pyloric region of the glandular stomach, less frequently in

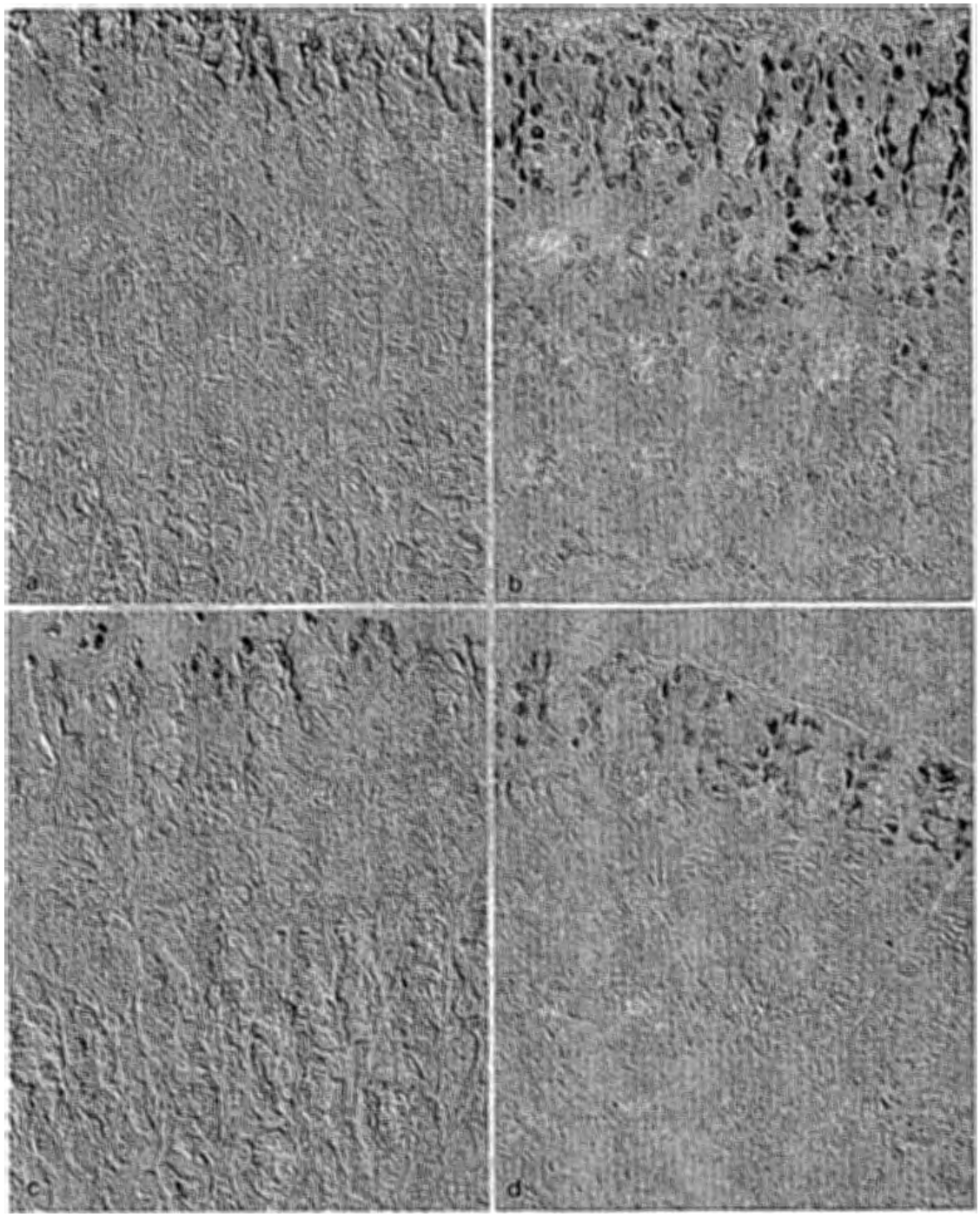

Fig. 1 a–d. Immunohistochemical demonstration of O^6-MedGuo in the fundic and pyloric mucosa of F344 rats following a single oral dose of 90 mg/kg *N*-methyl-*N*-nitrosourea (MNU) (**a, b**) and 1.3 mg/kg *N*-methly-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (**c, d**). In the fundus, the population of cells stained with anti-O^6-MedGuo was limited to the luminal surface after administration of both MNU (**a**) and MNNG (**c**). Note that in the pyloric mucosa, immunoreactivity following MNNG administration is restricted to a small layer of cells bordering the luminal surface (**d**), whereas after administration of MNU, the pyloric region contains immunoreactive cells also in the deeper mucosal layers (**b**). Normarski interference contrast microscopy (× 250)

the fundus and duodenum, and rarely in the other organs (Ohgaki and Sugimura 1988). Recently, it has been reported that MNU also induces gastric carcinomas when given in the drinking water in F344 rats. According to Maekawa et al. (1985), gastric carcinomas develop in about 40% of F344 rats exposed to MNU at a concentration of 100 ppm in the drinking water for 42 weeks. Hirota et al. (1987) reported that gastric carcinomas were selectively induced in 100% of F344 rats that were given MNU in the drinking water (400 ppm) for 25 weeks and then maintained without carcinogen for a further 20 weeks. Later, these authors found that administration

of MNU at 100 ppm for 15 weeks followed by 25 weeks without carcinogen was an optimal condition for the selective induction of gastric carcinomas in F344 rats without significant toxicity (Fujita et al. 1989).

In the present experiment oral administration of MNU for 2 weeks produced significant amounts of O^6-MedGuo in all rat tissues examined and no correlation was found between target organ specificity and the extent of DNA methylation. Highest amounts of O^6-MedGuo were found in the brain. This is due to the low activity of the repair enzyme, O^6-methylguanine DNA methyltransferase in the central nervous system (Kleihues and Margison 1974; Margison and Kleihues 1975) and correlates with the high incidence of brain tumours in F344 rats following chronic oral MNU administration (Maekawa et al. 1985). In the digestive tract, the forestomach showed a greater extent of DNA methylation than the other tissues, and oesophagus, pylorus and fundus of the glandular stomach and duodenum showed lower levels of O^6-MedGuo but they differed little between each other. This is in contrast to similar studies with MNNG, which showed O^6-MedGuo concentrations in the glandular stomach to be ten and four times higher than in forestomach and duodenum, respectively (Wiestler et al. 1983; Kobori et al. 1988). The high level of methylation in DNA of the target organ (glandular stomach) appears to be due to the presence of thiols. SH compounds (e.g. cysteine and reduced glutathione) accelerate the non-enzymatic decomposition and macromolecular binding of MNNG. In rats, concentrations of free thiols are considerably higher in the glandular stomach than in the oesophagus and forestomach (Boyd et al. 1979; Wiestler et al. 1983). At the same time, MNNG is rapidly degraded at the low pH of gastric juice (McKay and Wright 1947). This could explain the lower levels of methylation in duodenum, ileum and colon despite the high thiol concentration in these tissues (Boyd et al. 1979; Wiestler et al. 1983). In contrast, MNU is stable under the acidic conditions of the intragastric environment and its decomposition to a methylating intermediate, i.e. methyldiazonium hydroxide, is not affected by intracellular thiols. These characteristics might explain the widespread distribution of methylation in various tissues.

There is indirect evidence that the distribution of cells containing methylpurines within the gastric mucosa also differs between MNU and MNNG. After chronic administration of MNNG (80 ppm), cells staining for O^6-MedGuo were restricted to the luminal surface of the glandular stomach (Kobori et al. 1988). The amounts of O^6-MedGuo in inbred Wistar rats given MNNG at 80 ppm for 3–84 days were 50–100 μmol/mol guanine in pylorus and 20–50 μmol/mol guanine in the fundus and duodenum, respectively (Kobori et al. 1988). Although chronic exposure to MNU (400 ppm) produced similar levels of O^6-MedGuo in the pylorus and higher levels in other segments of the digestive tract of rats, no immunoreactive cells were detectable. This discrepancy is most likely due to a more uniform methylation of mucosal cells, i.e. the amounts of O^6-MedGuo present in individual nuclei are lower than the immunohistochemical detection limit. One explanation for a broader distribution of cells containing methylpurines is that intragastric MNU is not completely decomposed at the site of uptake into the gastric mucosa. In contrast to MNNG, a significant fraction of MNU is resorbed and redistributed via the bloodstream (Swann and Magee 1968; Swann 1968).

Our immunohistochemical studies showed that in rats given MNU as a single high oral dose (90 mg/kg), cells containing methylpurines were present in the surface epithelium of the fundus whereas, in the gastric pylorus, immunoreactivity to O^6-MedGuo and 7-MedGuo was also detectable in deeper layers of the mucosa. This phenomenon may explain the different susceptibility to MNU-induced carcinogenesis of pylorus and fundus (Hirota et al. 1987), i.e. tissues which in biochemical analysis showed similar levels of DNA methylation after administration of MNU for 2 weeks. If labelling of the deeper mucosal layers were due to systemically distributed MNU, such regional differences between pylorus and fundus would be unlikely to occur. It is more likely that MNU in the gastric lumen reaches deeper layers in the pyloric glands than in the fundic glands. Although no attempt was made to quantify the immunohistochemical data, it appeared that the staining in the fundic region of rats after a single oral dose of 90 mg MNU/kg was similar to that seen after a 70-times lower dose of MNNG (1.3 mg/kg body weight). No labelled cells were detected in any tissue of a rat given 20 mg MNU/kg (data not shown). These results also support the view that thiols enhance gastric DNA methylation by MNNG but not by MNU.

After a 2-week treatment with MNU, enhanced cell proliferation and histological changes were most pronounced in the forestomach and pylorus. Previous studies have shown that changes in the proliferative activity on the gastric mucosa may play a crucial role in the initiation of neoplastic transformation by MNNG in rats (Ohgaki et al. 1988, 1989). The combined data of DNA methylation and DNA synthesis suggest that forestomach and pylorus of the glandular stomach are at the highest risk of mutation during MNU treatment in the drinking water in F344 rats. However, resistance to MNU-induced carcinogenesis in the forestomach of F344 rats also suggests that genetic factors could significantly modulate MNU-induced gastric carcinogenesis in rats.

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