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Mechanisms of Differential Strain Sensitivity in Gastric Carcinogenesis

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The genetically-controlled, distinct sensitivity of different rat strains to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG)-induced cancer of the glandular stomach and duodenum was investigated. MNNG is activated through thiols, and the thiol content of the glandular stomach, duodenum, and liver of the BN rat tended to be slightly, but not significantly higher than that of the Wistar, Sprague-Dawley, Lewis, and Buffalo rats. The levels of the DNA repair system, O^6-alkylguanine transferase (AGT), in sensitive Wistar strain rats had values similar to those in resistant Buffalo strain rats. Administration of 80 mg/liter of MNNG in the drinking water for six weeks up to the time of tissue collection yielded the same AGT levels. Of all the parameters examined to account for genetically-mediated sensitivity to gastrointestinal cancer induction, namely, N-denitrosation, thiol activation, AGT-related DNA repair, and cell duplication rates, the latter yielded the best association, although these factors acting together may be involved.

Key words: Gastric cancer — N-Methyl-N′-nitro-N-nitrosoguanidine — Glutathione — O^6-Alkylguanine alkyltransferase — DNA synthesis and repair

After the development of an excellent model for glandular gastric cancer by Sugimura and Fujimura[1] that involves the simple administration of the direct-acting mutagen/carcinogen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) in drinking water to rats, Bralow *et al.*[2] and Martin *et al.*[3] discovered that there were large differences in sensitivity among various strains of rats. The random-bred Wistar rat and inbred BN rat were most sensitive, and the inbred Buffalo rat was resistant. Other strains had intermediary responsiveness (see ref. 4).

Recent studies suggest a continuing interest that has confirmed and extended findings. Inbred strains are often, but not always, less responsive than random-bred rats, for example, inbred Wistar/Furth, Buffalo, or Fischer F344 strain as compared with random-bred Wistar or Sprague-Dawley rats.[4–6] The inbred ACI, however, is quite sensitive.[7] The less sensitive Buffalo strain can develop gastric cancer if the period of observation, after limited intake of MNNG, is extended.[7]

The mechanisms underlying these distinct responses are not known but are important. Indeed, if different strains of rats display distinct sensitivity to a given carcinogenic regimen, the question of extrapolating the findings from laboratory models to human beings needs to be addressed (see refs. 7, 8). It seems likely that humans also have differential responses to a given carcinogenic environment. It would be useful to acquire an understanding of strain differences in the rodent system, therefore, as an approach to the development of specific tests for potential, distinct human sensitivities to a given carcinogenic environment.[9]

The reaction of MNNG with cellular nucleophiles is potentiated by thiols,[10, 11] since they react with the guanidine part of MNNG and liberate the reactive methyldiazonium hydroxide (see ref. 9). Thus, we determined the thiol concentration in the glandular stomach of rat strains with diverse sensitivities. DNA methylation by MNNG yields O^6-methylguanine, which has been implicated as a critical lesion in the initiation of tumors.[12–14] This adduct is repaired by the action of an alkyltransferase protein termed O^6-alkylguanine - DNA - alkyltransferase (AGT).[12, 15, 16] O^6-Methylguanine demethylation via the transferase reaction might account for distinct strain sensitivities, and this reaction was studied in two sensitive and resistant

strains, with and without pretreatment with MNNG.

Materials and Methods

Chemicals Reduced glutathione (GSH), glutathione reductase, reduced nicotinamide adenine dinucleotide phosphate and MNNG were purchased from Sigma Chemical Co. (St. Louis, MO). 5,5′-Dithiobis(nitrobenzoic acid) (DTNB) was acquired from Aldrich Chemical Co. (Milwaukee, WI).

Animals and Administration of MNNG Twelve-week-old male rats of the following strains were used: BN (Charles River, Wilmington, DE); Wistar (Mai/Hsd:WI-Br), Fischer F344, Sprague-Dawley and Lewis (all from Charles River, Kingston, NY), and Buffalo (Buf/N Hsd BR) (Harlan-Sprague Dawley, Indianapolis). Upon delivery from the supplier, rats were maintained in quarantine for 2 wk and appropriate health tests were performed in the Research Animal Facility (AAALAC accredited). Treatment and maintenance of animals conformed to the practices prescribed in the Guide For the Care and Use of Laboratory Animals.[17]

Assay for Thiols and Glutathione Three animals of each strain were used for every experiment, and each series of assays was done in duplicate. Animals were killed by decapitation, and the forestomach, glandular stomach, duodenum, and liver removed. Forestomach, glandular stomach, and duodenum were opened longitudinally, and food was removed by rinsing in four changes of ice-cold, phosphate-buffered saline. Mucosa of each organ were scraped with a glass slide, weighed, and homogenized, using a Tenbroeck tissue grinder, in 10 volumes of 2*M* perchloric acid containing 2m*M* EDTA. The homogenate was centrifuged at 3000*g* for 5 min and neutralized with an equal volume of 2*M* potassium phosphate buffer (pH 7.0). The extract was centrifuged at 3000*g* for 10 min. The supernatant was assayed for glutathione concentration. Liver was first minced with scissors and then extracted as above.

The glutathione assay was performed as described by Akerboom and Sies[18]; GSH reduces DTNB to a chromophore absorbing at 412 nm, and the disulfide is recycled to GSH by glutathione reductase. The thiol concentration in the extracts was determined by comparison with a standard curve prepared with GSH. Thiol concentrations were expressed as μmole GSH equivalents/g wet weight of tissue. Two replicate determinations were done for glandular stomach and duodenum in each experiment. Forestomach and liver, as reference tissues, were assayed only once per experiment.

Assay for O^6-Alkylguanine-DNA-alkyltransferase (AGT) Groups of 15 rats each of the Buffalo and Wistar strains were given freshly prepared solutions of 80 mg/liter of MNNG in dark water bottles that were changed twice a week. Control groups of 15 males of the Buffalo and Wistar strains were maintained on drinking water without MNNG. After 6 wk, these 60 rats were killed by a CO_2 atmosphere and dissected immediately. The forestomach, glandular stomach, duodenum, and liver, were removed and rinsed with 0.9% sodium chloride (isotonic saline) solution. The stomach and duodenum were leveled on a cork board and carefully scraped with a glass slide to obtain epithelial cells. The liver was grossly minced with scissors. The cells from stomach and duodenum and an aliquot from the liver were transferred to tared vials and immediately frozen on dry ice. These vials maintained in dry ice were shipped to the laboratory of Dr. A. Pegg at Hershey.

Tissues were stored frozen at $-70°$. For the tissues analyzed, samples from 3 rats in control group and 3 in the group given MNNG were pooled, and this usually yielded material for 5 AGT assays. Extracts containing AGT were prepared and AGT activity was assayed as previously described.[16, 19, 20] Results were expressed as units/mg of protein added where one unit of AGT catalyzes the removal of 1 fmol of O^6-methylguanine from the DNA substrate.

Results

Thiol and Glutathione Concentrations The thiol content of glandular stomach mucosa in the five strains of rat ranged from 0.50 to 0.81 μmol GSH equivalents/g of wet weight (Table I). Data for duodenum (0.92–1.2), forestomach (0.19–0.21), and liver (5.3–7.0) are presented in Table II.

Comparison of the thiol concentrations in glandular mucosa extracts among strains suggests that values in BN rats might be slightly higher and those in Sprague-Dawley rats somewhat lower. However, a two-way analysis of variance shows that differences among strains are only marginally significant ($0.05 < P < 0.10$).

The levels of glutathione in the forestomach were lower than in the glandular stomach (Tables I and II), but they were higher in the duodenum, and considerably higher in the liver. The values we found correspond to those reported by Wiestler *et al.*[11] in their Wistar strain.

AGT Activity A first set of experiments was done to establish the feasibility of extending previous findings of the level of AGT in rat

Table I. Thiol Content of Mucosa from Glandular Stomach of 5 Strains of Rats[a]

Experiment series	BN	Wistar	Sprague-Dawley	Lewis	Buffalo
Series #1	0.75±0.0[b]	0.53±0.11	0.45±0.0	0.53±0.15	0.57±0.17
Series #2	0.87±0.0	0.81±0.04	0.54±0.21	0.69±0.0	0.65±0.06
Averages	0.81±0.07	0.67±0.18	0.50±0.13	0.61±0.11	0.61±0.11

a) A 65 μl aliquot (3.3 mg wet weight) of extract was assayed in duplicate for each experiment. Values shown are means and standard deviations in μmol GSH equivalents/g wet weight.
b) The standard deviation reads 0.0 where the results obtained in two experiments, each in duplicate, were identical.

Table II. Thiol Content of Extracts from Duodenal Mucosa, Forestomach Epithelium and Liver in 5 Strains of Rats[a]

Site	BN	Wistar	Sprague-Dawley	Lewis	Buffalo
Duodenum	1.2±0.11	1.04±0.09	0.92±0.27	1.02±0.1	1.01±0.16
Forestomach	0.19±0.14	0.20±0.06	0.21±0.04	0.21±0.42	0.21±0.04
Liver	7.0±0.84	6.2±0.84	5.7±0.42	5.3±0.7	5.3±0.98

a) Volumes assayed were duodenum, 50 μl (2.5 mg wet weight); forestomach, 100 μl (5 mg); liver 10 μl (0.5 mg). Values are mean and standard deviations from duplicate experiments in μmol GSH equivalents/g wet weight.

Table III. AGT Activity in Extracts from Duodenum, Forestomach, Glandular Stomach and Liver

Tissue	Strain	Treatment	AGT activity[a] (units/mg/protein)
Duodenum	Wistar	None	1.8
Duodenum	Wistar	MNNG[b]	2.0±1.0
Duodenum	Buffalo	None	1.2
Duodenum	Buffalo	MNNG	1.6±0.3
Forestomach	Wistar	None	7.6±1.1
Forestomach	Wistar	MNNG	5.5±0.8
Forestomach	Buffalo	None	9.0±2.1
Forestomach	Buffalo	MNNG	7.5±0.7
Glandular stomach	Wistar	None	1.3±0.4
Glandular stomach	Wistar	MNNG	0.9±0.5
Glandular stomach	Buffalo	None	1.3±0.3
Glandular stomach	Buffalo	MNNG	1.4±0.6
Liver	Wistar	None	43±13
Liver	Wistar	MNNG	47±6
Liver	Buffalo	None	60±11
Liver	Buffalo	MNNG	47±11

a) The results shown are the mean±SD for 4 or 5 estimations, usually the result of the pooled tissues of 3 rats each (see text). Where no SD is shown the results are the mean of 3 estimations.
b) A solution of 80 mg/liter MNNG was freshly prepared twice a week and offered to rats in dark glass bottles for 6 weeks right up to time of killing and tissue preparation. The Wistar and Buffalo rats had an average daily water intake of 26 ml and 21 ml per rat, respectively.

liver to tissues of relevance in the present report, namely, forestomach, glandular stomach, and duodenum. It was found that the levels in the upper intestinal tract of F344 rats were readily measured but were considerably lower than the levels prevailing in rat liver

(data not shown). Concurrent administration of MNNG did not influence the concentration of AGT in the stomach or duodenum, or in the liver, which is a control tissue since MNNG as such is not expected to reach the liver in significant amounts, compared with the stomach or duodenum.

Examination of the level of the enzyme in the MNNG-sensitive Wistar strain vs. the resistant Buffalo strain indicated that there were minimal differences between the two strains (Table III). The liver had the highest levels of activity per mg of tissue of the organs studied. The concentrations in the forestomach in turn were higher than those in the glandular stomach. The levels in the duodenum were similar to those in the glandular stomach (Table III). Animals given MNNG in the drinking water for 6 wk right up to the time of necropsy had levels identical to control animals, not only in the liver but also in the forestomach, glandular stomach, and duodenum.

Discussion

A series of studies was conducted in rat models for glandular stomach cancer to generate information bearing on strain differences in response. It was established that distinct strains of rats had different incidences of gastric cancer following identical treatment with MNNG in drinking water.[3-7, 21] The randomly bred Wistar rats and the inbred BN strain rats had the highest sensitivity; the inbred rat of the Buffalo strain had the lowest sensitivity. One approach to explore the mechanisms of the genetically controlled sensitivity was based on the fact that nitrosamides and nitrosamines could be detoxified by denitrosation (see refs. 8, 22). A first set of studies examined whether differential strain sensitivity could be accounted for by biochemical denitrosation since loss of the nitro group would eliminate carcinogenicity. While there were differences in denitrosation, the association with sensitivity as a function of strain was weak.[8]

Therefore, we conducted the additional studies reported herein. Previous reports indicated that thiols could exert an enhancing effect on the reactivity of alkyl nitrosamides such as MNNG to nucleophiles such as DNA,[10, 11] an effect distinct from the protective effect exerted by GSH and GSH transferase against other toxic agents such as ethanol[23] and carcinogens.[24, 25] Thus, it seemed relevant to determine thiol concentrations in the stomach of sensitive and resistant strains. As was reported recently,[26] we also noted a lower level of nonprotein sulfhydryls in the stomach and duodenum, compared to liver. Also, the thiol content of glandular stomach, duodenum, and liver of the sensitive BN rat was slightly greater than that of the Wistar, Lewis, and Buffalo rat strains. The differences, however, were not significantly different in all tissues examined of the five strains studied. Thus, this particular parameter by itself is not able to account for the decisive differences in carcinogenic response.

The groups of Kleihues, of Rajewsky, of Pegg, and others have established that certain organ-specific actions of alkyl nitrosamides could be explained neatly by the amount of available alkyl transferase in a given tissue (see refs. 16, 27–30). For example, rat liver had high amounts of this enzyme, kidney less, and brain had low amounts. Administration of nitrosomethylurea fails to induce primary liver cancer, yields a smaller incidence of renal cancer, but affects primarily the brain, demonstrating an inverse association with the amount of alkyltransferase in these tissues. In turn, these findings are interpreted in terms of DNA repair being high in liver, appreciable in kidney, and minimal in brain. It seemed, therefore, that the O^6-methyl transferase, acting as a DNA repair system specifically after the action of methylating agents such as MNNG, might account for strain differences, particularly in the upper gastrointestinal tract. Also, depletion of AGT increased mutagenic responses in cell culture systems,[31] and under other cell culture conditions, MNNG itself depleted AGT.[32] In the present study, two important findings were made: 1, in all organs studied, including glandular stomach and duodenum, target organs of oral MNNG, the tissues of a sensitive strain had the same level of AGT as those of a resistant strain; and 2, administration of MNNG right up to the time of tissue harvest gave the same levels of AGT as in naive controls. Relevant is the finding that isotope from labeled MNNG was found to the same extent in the stomach mucosal DNA of a sensitive and of a resistant strain.[33]

Even if the three approaches discussed herein, namely, differential denitrosation of MNNG, thiol concentrations in the stomach and duodenum, and alkyl transferase are examined together, since each element may not be independent of the others, the battery of data adduced thus far does not account fully for the appreciable, distinct responses to oral intake of this carcinogen. Thus, other hypotheses need to be tested on this important difference in strain-related sensitivity in gastric carcinogenesis.

Similar findings of complex biochemical effects were made in accounting for strain and species differences in colon carcinogenesis with 1,2-dimethylhydrazine and the metabolite azoxymethane.[34, 35] A promising lead was produced by Deschner,[36] who found that the rate of thymidine incorporation reflecting DNA synthesis in the colon stem cells agrees reasonably with the strain sensitivity in mice. Likewise in humans, Lipkin[37] among others, has information that families at higher risk of genetically-mediated polyposis display different cell parameters from controls. There may be merit, therefore, in accounting for strain and species differences in the upper gastrointestinal tract through the determination of DNA synthesis and mitotic rates[38] or through fixation of DNA strand breaks.[35] A recent paper confirmed that rat strains of differing sensitivities to MNNG display similar levels of label from MNNG in gastric cells. However, the important finding was made that the rate of cell proliferation, measured by bromodeoxyuridine (BrdU) incorporation, is higher in the more sensitive rat strain.[39] Thus, cell duplication rates may be key indicators of sensitivity to carcinogens not only in the colon but also in the glandular stomach.

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