[CANCER RESEARCH 39, 2733–2736, July 1979]
0008-5472/79/0039-0000$02.00

Gastrointestinal Carcinogenesis in Germ-free Rats Given *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine in Drinking Water[1]

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ABSTRACT

This study was designed to clarify the role of gut microflora in tumorigenesis by a comparison of tumor production between male germ-free and conventional Wistar rats given *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), 100 μg/ml in drinking water.

Ninety-one % of conventional MNNG-treated rats that died or were killed by Day 314 of the experiment developed tumors in the gastrointestinal tract, whereas only 17% of germ-free treated rats developed such tumors. In addition, large tumors, some 5 cm or more in diameter, were frequently observed in the conventional rats, whereas only small tumors 0.4 to 1.2 cm in diameter were present in the germ-free rats. Furthermore, multiple tumors including double tumors were often found in the conventional rats, while such tumors never appeared in the germ-free rats.

The results suggest that gut microflora might exert a promoting influence on tumorigenesis by MNNG in the gastrointestinal tract. The promoting influence of the microflora in conventional rats might not be of a simple nature, since the influence of a variety of factors modified by the microflora on tumorigenesis by MNNG p.o. is unavoidable.

INTRODUCTION

Sugimura and Fujimura (13) reported that, after 12 months of continuously receiving MNNG[3] dissolved in drinking water at a concentration of 33 or 83 mg/liter, rats developed neoplastic lesions in the glandular stomach with very high frequency. Bralow *et al.* (4) reported that, when MNNG was given continuously in drinking water at a concentration of 83 mg/liter, 60% of the rats developed tumors by the 41st week and 80% had gastric adenocarcinomas by the 52nd week. Later, it was reported by a number of investigators that varied tumors were produced in the gastrointestinal tract of animals of several laboratory species that were given MNNG in drinking water (6, 12). Many microorganisms are lodged in the gastrointestinal tract and are presumed to have a close relationship with the host. Therefore, we became interested in the role of the microflora in carcinogenesis in the gastrointestinal mucosa. This paper deals with a comparison of the production of tumors in germ-free and conventional rats that were given similar amounts of MNNG p.o.

MATERIALS AND METHODS

Male germ-free and conventional Wistar rats, weighing 80 to 95 g at the start of the experiment, bred in the Laboratory of Germfree Animal Research, Kawashima, Gifu-ken, Japan, were used. The germ-free rats were maintained, 3/cage, in a type MS-66 stainless steel isolator (8). The germ-free status was checked once every 2 weeks as described by Wagner (15). The conventional rats were housed, 3/cage, in a temperature- and humidity-controlled clean room. The composition of the diet was described previously (8). The diet was steam-sterilized at 121° for 25 min and was given *ad libitum* to both experimental and control rats. MNNG (Aldrich Chemical Company, Inc., Milwaukee, Wis.) dissolved in drinking water at a concentration of 100 μl/ml was continuously given *ad libitum* to experimental groups until the termination of the study. According to the report of Sugimura (14), MNNG in the stock solution is stable for at least 1 week, and MNNG in the diluted solution is stable for at least 2 days. Therefore, the stock and diluted solutions were prepared as follows: (*a*) the stock solution was prepared by dissolving 4 g of MNNG in 1000 ml of distilled water. The solution was freshly prepared once a week; (*b*) the stock solution was passed through a Millipore filter into 2 sterilized containers. The containers were introduced into an autoclave attached to the isolator, and their surfaces were sterilized under peracetic acid spray. Then one container was transferred into the isolator for germ-free rats, and the other was taken out of the autoclave for conventional rats; (*c*) the filtered stock solution of MNNG was diluted with steam-sterilized tap water to obtain drinking water at a concentration of 100 μg/ml. The diluted water was replaced with new MNNG-containing water once every 2 days. The bottles with the diluted water were covered with aluminum foil to protect MNNG from degradation by light. The concentration of MNNG in both the solution kept in the germ-free isolator and the solution kept in the conventional room was occasionally checked by a spectrophotometry at the end of a 2-day period; there was no significant difference between the 2 samples. The average dosage of MNNG intake per rat per day was 2.3 ± 0.36 (S.D.) mg in germ-free treated rats and 2.2 ± 0.41 mg in conventional treated ones. Ten germ-free rats and 10 conventional rats were supplied with the sterilized drinking water without MNNG as controls.

Nineteen conventional treated rats either were found dead or were killed in moribund condition from tumor by Day 313 of the MNNG treatment, whereas none of germ-free treated rats died or were moribund from tumor by the same date. However, 4 rats died or were moribund from volvulus due to a big cecum characteristic of the germ-free state. All the surviving treated rats (26 germ-free and 14 conventional) were sacrificed on Day 314, together with control rats not treated with MNNG. All

[1] This work was supported in part by a Grant-in-Aid for Cancer Research from the Japan Ministry of Education.

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[3] The abbreviations used are: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; i.r., intrarectal.

Received November 3, 1977; accepted January 22, 1979.

rats that died or were killed were autopsied and examined for tumors on various organs, including the entire gastrointestinal tract. For light microscopy, tissues were fixed in 10% formaldehyde solution and embedded in paraffin. Sections were stained with hematoxylin-eosin and by the Van Gieson and periodic acid-Schiff methods.

RESULTS

There was a remarkable difference in tumor incidence between germ-free and conventional MNNG-treated rats. Only 17% of the germ-free treated rats had tumors, whereas 91% of the conventional treated rats had tumors (Table 1). None of the germ-free and conventional rats used as controls had tumors. One germ-free treated rat (3.3%) developed an adenocarcinoma. The tumor, found in a rat killed in moribund condition due to volvulus on Day 281, extended from the duodenum to the pyloric valve (Fig. 1). It was found as a nodular growth, 0.9 cm in diameter, elevated on the mucosal surface. On the other hand, adenocarcinomas were found in 14 conventional treated rats (42%). With the exception of 2 in the stomach, the tumors were localized almost exclusively in the duodenum and jejunum. The average latent period of the tumor was 286 days, the first tumor being found on Day 245. They varied in diameter from 0.8 to 3.2 cm and were nodular, papillary, and sometimes ulcerated in shape. Microscopically, the tumors of both germ-free and conventional groups had glandular structures and extended to the submucosa and muscularis, sometimes infiltrating into the serosa (Fig. 2). However, more anaplastic cells and mitoses were found in the conventional rats than in the germ-free group. A leiomyosarcoma was found in one germ-free treated rat (3.3%) killed on Day 314 and was localized in the duodenum 3.2 cm from the pyloric valve. It was a firm nodular growth, 1.2 cm in diameter (Fig. 3). In contrast, leiomyosarcomas were found in 11 conventional treated rats (33%). They often appeared during the early period of the experiment. The average latent period of the tumors was 247 days, the first being found on Day 190. Their rapid growth often led to necrosis in the central portion of the tumor. Microscopically, the tumors of both groups consisted of intertwining bands of spindle-shaped cells (Fig. 4). However, the tumor cells of the conventional rats demonstrated more nuclear and cytoplasmic polymorphisms than those of the germ-free rats. Hemangioendotheliomas were found in 2 germ-free treated rats (6.7%). In one case, the tumor, a red soft nodular growth, 0.5 cm in diameter, was localized in the duodenum 6.2 cm from the pyloric valve. The other hemangioendothelioma was localized in the duodenum, 2.3 cm from the pyloric valve. It was a nodular growth, soft in part, firm in part, and 0.5 cm in diameter (Fig. 5). Hemangioendotheliomas were found in 4 conventional treated rats (12%). They were generally small, nodular, dark-reddish growths and were located near the serosal portion of the duodenum and jejunum. Microscopically, the tumors of both groups were composed of vascular spaces varying in shape and size, and they sometimes showed a projection of the proliferating endothelium into the vascular spaces, there being no difference in the morphological pattern between the germ-free and conventional groups (Fig. 6). Only one conventional treated rat had a carcinosarcoma, which was a mixed carcinomatous and sarcomatous growth. Besides the tumors described above, we found adenomas in one germ-free rat and in 6 conventional rats, all characterized by cellular components resembling normal original cells and by an expansive growth.

With regard to lesions in organs other than the stomach and small intestine, one hepatocellular carcinoma and 2 hepatocellular adenomas were observed only in conventional rats. Multiple tumors, including double tumors, were observed in 8 conventional treated rats (24%), but such tumors were not observed in germ-free rats (Fig. 7).

Table 1

Incidence and structural classification of gastrointestinal tumors in germ-free and conventional rats given MNNG (100 μg/ml) in drinking water until Day 314

	Germ-free rats with tumor		Conventional rats with tumor	
	No.	%	No.	%
Incidence	5/30[a]	16.7	30/33	90.9
Structural classification[b]				
Adenocarcinoma	1	3.3	14	42.4
Leiomyosarcoma	1	3.3	11	33.3
Carcinosarcoma	0	0	1	3.0
Hemangioendothelioma	2	6.7	4	12.1
Adenoma	1	3.3	6	18.2

[a] Number of rats with tumors/number of rats examined.
[b] Including rats with a double tumor.

DISCUSSION

Our data demonstrate well that the gut microflora might have exercised a promoting influence on tumorigenesis in the gastrointestinal tract of rats that received MNNG in drinking water. Regarding the production of colonic cancer in germ-free animals, Reddy *et al.* (10) reported that 100% of both germ-free and conventional rats that received weekly i.r. injection of 1 to 3 mg MNNG per rat for 20 weeks and were autopsied 30 weeks after the last injection had tumors. However, MNNG injection nearly doubled the multiplicity of colonic tumors (number of tumors per rat) in germ-free rats compared to conventional rats. They did not find such distinct differences between the 2 groups as we did. The discrepancy might be attributed to the following issues: (*a*) the difference in the treatment technique. In the one experiment, MNNG was given *ad libitum* at a concentration of 100 μg/ml in the drinking water, whereas it was injected i.r. in doses of 1 to 3 mg per rat in the other experiment; (*b*) the difference of the target organ in tumorigenesis, being the stomach and duodenojejunum in our study and the colon in the study of Reddy *et al.*

Since MNNG is generally accepted to be a direct-acting carcinogen, it was first thought that the microflora must have had a promoting effect on the development process after the initiation of the tumor in the gastrointestinal tract. This explanation raises the question as to whether the microflora also has such a promoting effect on nonepithelial tumors, from the point of view of the theory of Berenblum (2). At this juncture, the possibility that the turnover of cells in the lamina propria of the small intestine is affected by the living flora in the same way as the intestinal epithelium was indicated (1), suggesting the influence of the flora on the cells of the mesenchymal tumor arising from the lamina propria. All leiomyosarcomas in this experiment were produced in the mucosa and were in close relation with

the gut microflora. Therefore, it is possible that the gut microflora exercises some promoting effects on the development of nonepithelial tumor in the mucosa, including the muscularis mucosae, with the exception of the hemangioendothelioma which had no connection with the mucosa. In connection with the above, it is noted parenthetically that Narisawa *et al.* (9) reported that some bile acids acted as promoters of MNNG-induced colon carcinogenesis in the presence of the intestinal flora.

Another point requiring consideration is the degradation of MNNG. We noted that the preparation and preservation of the MNNG solutions before the treatment were done under almost the same conditions in both germ-free and conventional groups described above. Granting this, the metabolism *in vivo* of MNNG p.o. cannot be neglected. It is presumed that MNNG is decomposed rapidly to *N*-methyl-*N'*-nitroguanidine in acidic conditions (7). Because of this, the difference in the disposal of MNNG in gastric juice between germ-free and conventional rats will be a matter of concern. Furthermore, there have been several previous investigations on the differences in the digestive mechanisms of the gastrointestinal tracts of germ-free and conventional rats. The rate of turnover of ileal epithelium in the germ-free status was found to be significantly lower than in the presence of the conventional flora (1). Considering the longer life span of intestinal cells in germ-free rats, the activities of the mucosal enzymes are of a great interest. One or several of the normal microbiological inhabitants of rat intestine were inferred to be responsible for normally occurring inactivation of the digestive enzymes of intestinal contents (3). Activities of some enzymes of mucosal extracts in the small intestine were shown to be increased in germ-free rats or to be significantly higher in germ-free growing rats than in conventional ones (5, 11). These data might suggest some explanation for the low incidence of tumor production in germ-free rats if any mucosal enzymes were concerned with the degradation of MNNG.

From those items described above, it can be speculated that there is some difference between germ-free and conventional rats in mucosal behavior against MNNG p.o. However, we are not quite sure whether the gut microflora exerted a promoting effect on the development process of gastrointestinal tumors induced by MNNG or whether the microflora exerted some promoting influences on the mucosal tumorigenesis through varied factors modified by its presence. That is, we presume that the promoting effect of the microflora on the tumorigenesis in the gastrointestinal tract is not of a simple nature.

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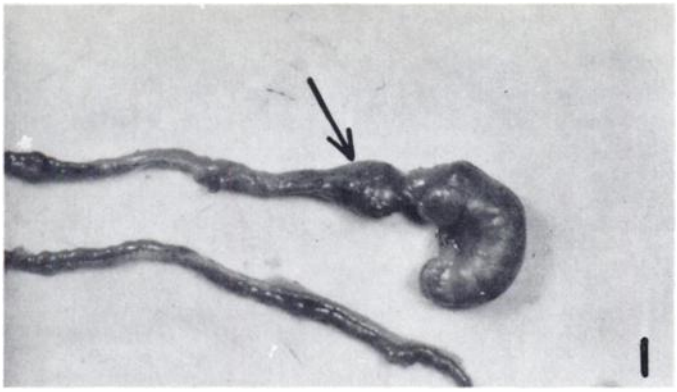

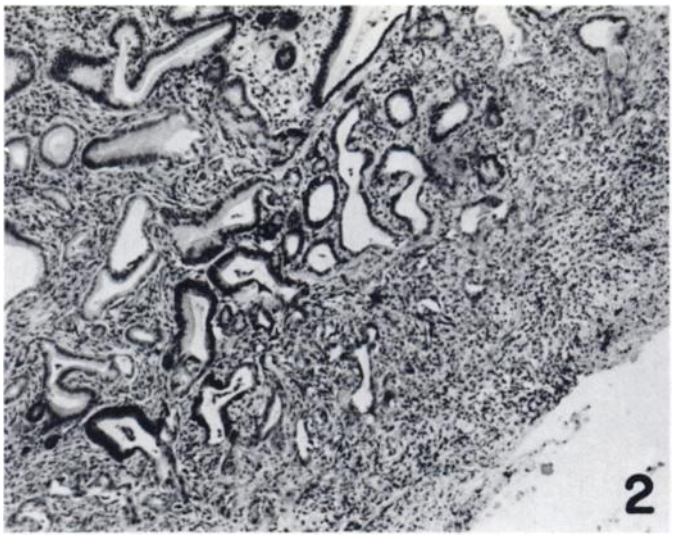

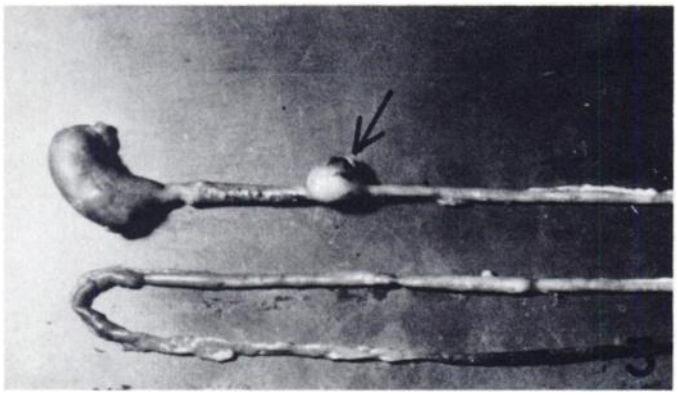

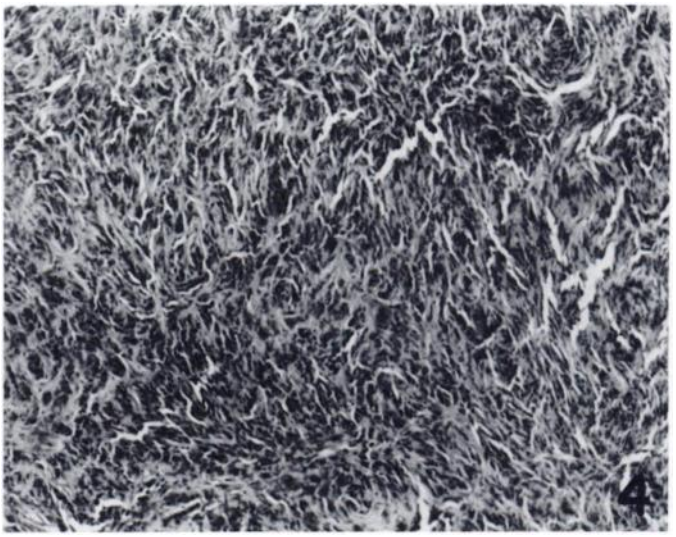

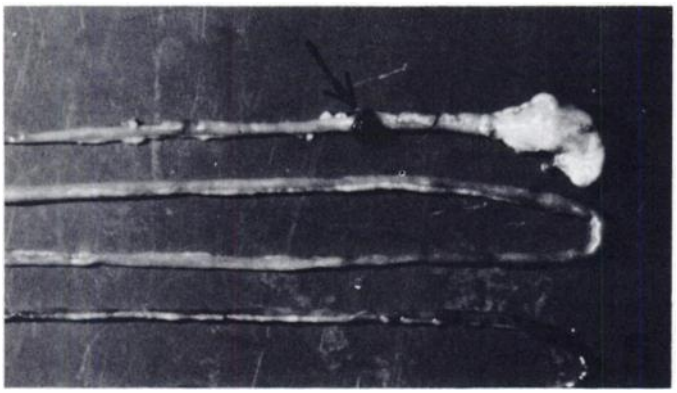

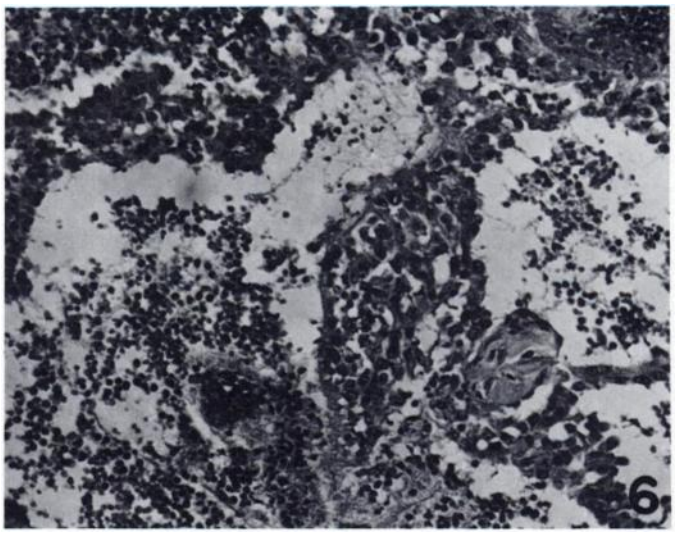

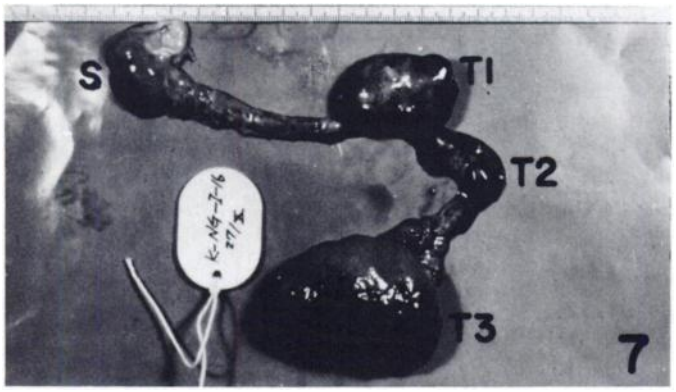

Fig. 1. Gross aspect of an adenocarcinoma observed in the duodenum of a germ-free rat killed on Day 281 of the MNNG treatment.

Fig. 2. Microphotograph of the adenocarcinoma shown in Fig. 1. The tumor extends through the submucosa and muscularis, partly involving the serosa. H & E, × 120.

Fig. 3. Gross aspect of a leiomyosarcoma observed in the duodenum of a germ-free rat killed on Day 314 of the MNNG treatment.

Fig. 4. Microphotograph of the leiomyosarcoma shown in Fig. 3 and composed of bundles of spindle-shaped cells. H & E, × 300.

Fig. 5. Gross aspect of hemangioendothelioma observed in the duodenum of a germ-free rat killed on Day 314 of the MNNG treatment.

Fig. 6. Microphotograph of the hemangioendothelioma shown in Fig. 5. Composed of vascular spaces. Note the proliferation of endothelial cells lining the vascular space. H & E, × 300.

Fig. 7. Gross aspect of multiple tumors found in a conventional rat killed on Day 314 of the MNNG treatment. *S*, stomach; *T1*, leiomyosarcoma; *T2*, hemangioendothelioma; *T3*, leiomyosarcoma.