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Cell Proliferation and Growth of Gastric Carcinoma Induced in Inbred Wistar Rats by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine[1]

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ABSTRACT

Gastric carcinoma was induced in inbred Wistar rats by p.o. administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine for 25 weeks, and cell proliferation and growth of the gastric carcinoma in an incipient stage were studied. A microscopic cancer was found by 24 weeks, and macroscopic cancers were found after 27 weeks. All the cancers were a single lesion located at the midpoint of the lesser curvature of the stomach. Histologically, they were tubular adenocarcinomas. The mucosal changes predisposing to the development of carcinomas were focal erosions and dysplasias confined to the midpoint of the lesser curvature. The malignant transformation appeared to occur in the dysplastic cells of the eroded mucosa by 17 to 18 weeks after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. Following the malignant change, the labeling indices of the tissues with [^{3}H]thymidine decreased, suggesting an elongation of cell cycle time. By repeated injections of [^{3}H]thymidine, a time required for all the cancer cells to enter S phase (reflecting the maximum cell cycle time) was estimated to be about 3.5 days. This gave a theoretical doubling time for the gastric cancers. On the other hand, from the temporal observations of tumor volumes, it was shown that the gastric cancers in an incipient stage underwent exponential growth with a doubling time of 14 days. The difference between the theoretical and actual doubling time might reflect a cell loss rate in the cancer tissue.

INTRODUCTION

Gastric cancer is now easily induced in the stomach of various experimental animals by MNNG[3] (13, 14). Several investigators have reported the temporal histological changes seen at various intervals during the development of MNNG-induced gastric cancers (3, 13). The administration of MNNG first causes superficial mucosal damage. As a result of this erosion, a process of repair is initiated, followed by regenerative hyperplasia. With continued treatment, adenomatous lesions arise, and thereafter early and invasive carcinomas are seen (3). Whether the emergence of a particular lesion can mean that it originates from a previous stage of atypical histology is still open to question. Since it is impossible to follow the particular lesions in the same rat, such studies must be done using many animals, but most lesions usually arise at different sites of the stomach, making their observation in chronological order difficult (3, 13). In 1976, however, Kobori *et al.* (8), using inbred Wistar rats, were successful in inducing the erosion, the adenomatous hyperplasia and the carcinoma, always located at the midpoint of the lesser curvature. With this experimental model, a detailed study of the development of gastric carcinomas seems to be possible. This study was undertaken to elucidate the growth rate of the gastric carcinoma in an incipient stage. Special attention has been focused on the identification of minute cancers. For this purpose, inbred Wistar rats were given MNNG for a relatively short period, and mucosal lesions at earlier stages, their sequential changes, and changes of the tumor volume were studied. For the identification of carcinomas, particularly in their early stages, labeling patterns and labeling indices of the tissue after a single injection of [^{3}H]thymidine were evaluated.

MATERIALS AND METHODS

Administration of MNNG and Sampling. The animals used in this investigation were 75 male Wistar rats, weighing 180 to 200 g. They were obtained from the Institute of Experimental Gerontology in Basel and inbred at the Institute of Pathology, University of Bonn, since 1961 (about 50 generations). MNNG was diluted with distilled water at a concentration of 80 μg/ml. Following Sugimura's method (14, 15), the solution was stored in a black bottle to avoid the degradation of MNNG by light and was changed every other day. In order to observe minute cancers in an incipient stage and to follow their growth as precisely as possible, administration of MNNG was limited to a short period. For 25 weeks, 60 rats were allowed to drink only this MNNG solution *ad libitum*. From the 26th week, they were given tap water. The remaining 15 rats, given only tap water, served as controls. Commercial food was given to all the rats. From the 9th to the 45th week, 4 of the MNNG-treated rats were sacrificed every 3rd week in order to examine sequential changes in the gastric mucosa and tumor volumes. At the 48th and 51st weeks, respectively, 4 rats were killed, from which tumor volumes and modes of increase in labeling indices with [^{3}H]thymidine were studied.

Autoradiographic and Histological Studies. The MNNG-treated rats killed from 9 to 45 weeks were given a single i.p. injection of [6-^{3}H]-thymidine ([^{3}H]thymidine; specific activity, 20 Ci/mmol; Radiochemical Centre, Amersham, England) at a dose of 1 μCi/g body weight 45 min before sacrifice. From these rats, the density and the distribution of the proliferative cells in the gastric mucosa and in the cancer tissue were studied. Five control rats were killed at the 21st, 30th, and 51st weeks, respectively, 45 min after a single injection of [^{3}H]thymidine. At the 48th week, 4 MNNG-treated rats received 4 repeated injections of [^{3}H]thymidine at 8-hr intervals (cumulative labeling for 24 hr). They were killed 45 min after the last injection. At the 51st week, 4 rats were given 7 repeated injections of [^{3}H]thymidine at 8-hr intervals (cumulative labeling for 48 hr), and they were killed 45 min after the last injection. All the rats were killed at 10 a.m. to avoid diurnal variations in labeling index.

The removed stomach was opened along the greater curvature, and the mucosal surface was then washed with 0.9% NaCl solution. The stomach was pinned out flat on a cork block and fixed in 10% formalin for 48 hr. After fixation, gross examination of the gastric mucosa was done with a dissecting microscope, and a photograph of each fixed rat stomach was taken. Since all lesions such as erosions and neoplasms were confined to the midpoint of the lesser curvature (8), the stomach was cut into 2 pieces along the lesser curvature and embedded in

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[3] The abbreviation used is: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

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paraffin. They were carefully oriented and sectioned serially. Sections about 5 μm thick were made. They were mostly stained with hematoxylin and eosin. Some sections were stained with periodic acid-Schiff. From observations of serial sections, the volume of the cancer in each stomach was set as follows. In light microscopy, the length, width, and thickness of the neoplastic lesions were measured. Since all the cancers were oval or spherical (9), the cancer volume was set by computer, assuming that the cancer might be a sphere with a diameter of the arithmetic mean of these 3 parameters. All the data were plotted semilogarithmically. The growth curve of the tumors was obtained by an exponential curve fit by HP 85 computer (Hewlett Packard Company, San Diego, CA).

Sections of the MNNG-treated stomachs for autoradiography were taken at 100-μm-thickness intervals. These skipped sections taken from each MNNG-treated rat and sections from control rats were dipped in Sakura NR-M2 photographic emulsion (Konishiroku Photo Ind. Com., Ltd., Tokyo, Japan), developed after 5 weeks of exposure, and stained with hematoxylin and eosin. In our autoradiographs, the background grain count was very small (less than 10 grains/0.01 sq mm), and cell nuclei were identified as labeled when they were covered with more than 10 grains. In the case of the normal antral mucosa, the labeling index was determined as follows. Square areas consisting of the proliferative cell zones of several pyloric glands were randomly selected under a microscope, and the percentage of labeled cells in the proliferative cell zone was calculated for each square area. The proliferative cell zone was considered as the part of the tubule between the "uppermost" and "lowermost" labeled cells, respectively. In measuring the labeling index of the eroded mucosa, its compatibility was taken into account. In the eroded mucosa, the labeled cells were widely distributed from the surface to the depth of the mucosa. In this case, however, we counted the percentage of labeled cells in the middle third level of the mucosa, which corresponded to the original proliferative cell zone of normal pyloric mucosa. This served to avoid the effect of reactive proliferation of mature cell types to the labeling index, and the obtained index could mean a ratio of DNA-synthetic cells to all the proliferative cells. In most of the dysplastic and neoplastic lesions, the labeled cells were found randomly throughout the tissue. The random distribution of the labeled cells might imply that the lesions consisted exclusively of proliferative cells. When the tissues were composed of proliferative cells, their labeling indices could be comparable to each other. In the dysplastic and neoplastic lesions, the square areas were randomly selected under a microscope. The labeling indices of 18 to 56 square areas were combined to obtain a final labeling index in the normal antral mucosa, in the eroded mucosa, in the dysplastic lesions, and in the neoplastic lesions. The total number of counted cells was more than 3000 in each case. In terms of these labeling indices, different kinds of lesions were compared to each other. The quantitative difference between the labeling indices was assessed by Student's *t* test.

By the cumulative labeling experiment, a time required for all the cancer cells to enter S phase was estimated, assuming the cells to be uniformly distributed in the cell cycle. This gives a maximum cell cycle time. The fraction of cells in S phase in MNNG-induced cancers was known after a single [^{3}H]thymidine injection. In this study, 3 labeling indices of the cancers were randomly chosen from the rats killed from 36 to 45 weeks to obtain a final labeling index to represent the fraction of cells in S phase. The labeling indices of cancer tissues after 4 and 7 repeated injections of [^{3}H]thymidine at 8-hr intervals, calculated at the 48th and 51st weeks of MNNG treatment, respectively, might include the increase of the labeled cells in every 24 hr. When there is no further increase in labeling index (in this study, this is 100% labeling index), the time elapsed would be the sum of $T_{G_1} + T_{G_2} + T_M$, *i.e.*, $T_C - T_S$ (4). With these 3 labeling indices, T_C was determined by a linear regression analysis with HP 45 and HP 85 personal computers (Hewlett Packard Company).

RESULTS

Macroscopic Findings. Early changes in the gastric mucosa induced by MNNG were erosions 0.2 to 0.4 cm in diameter. The erosions were always found at the midpoint of the lesser curvature in all rats treated with MNNG for 9 to 21 weeks (Fig. 1*A*). They were a single lesion. Other parts of the mucosa were not involved. After 27 weeks, macroscopic cancers were found at the midpoint of the lesser curvature (Fig. 1, *B* to *D*). They were also a single lesion. The neoplastic lesions showed a central ulceration and marginal thickening. The sizes of the neoplastic lesions varied among the simultaneously killed animals, but the lesions appeared to become large with time. The control rats showed no remarkable changes in the stomach.

Histological and Autoradiographic Findings. The control rats disclosed no histological abnormalities. In these rats, the labeled cells with [^{3}H]thymidine were confined to the middle level of the mucosa; *i.e.*, the neck region (Fig. 3*A*), and the labeling index was an average of 30.6% (Table 1). The erosions found in MNNG-treated rats killed from 9 to 21 weeks showed several different depths; in most cases, the surface of the mucosa was eroded and regenerating epithelium covered the surface incompletely (Fig. 2), while in some cases the mucosa was deeply eroded and its surface was covered with a huge amount of fibrin and leukocytes. The lamina propria of the eroded region was edematous, and fibrous tissue increased. The regenerating cells had relatively basophilic cytoplasm, and their nuclei were round. The labeled cells, after a single injection of [^{3}H]thymidine, were widely distributed from the surface to the bottom of the erosive mucosa (Fig. 3*B*). The labeling indices of the tubules located at the middle third level of the eroded mucosa were around 44.3% (Table 1).

In a rat killed at the 18th week and in 2 rats killed at the 21st week, irregular glandular tubules were found within the eroded mucosa at the midpoint of the lesser curvature. Some of the tubules were dilated and were composed of dysplastic cells, which contained basophilic cytoplasm and deeply stained, irregularly shaped nuclei. The cellularity was high in these glands, and a moderate cellular atypism was seen. However, it was difficult to diagnose these lesions as neoplasms. Their labeling index was an average of 24.0% (Table 1). In one rat, small atypical tubules were seen in the submucosa, in which [^{3}H]-thymidine-incorporated cells were randomly scattered (Fig. 3*C*).

In 2 rats killed at the 24th and 27th weeks, small dysplastic tubules were seen in the submucosal layer. They consisted of a homogeneous population of mucus-depleted cuboidal cells; they had basophilic cytoplasm and irregularly shaped nuclei. The location of these lesions was also at the midpoint of the lesser curvature. The mucosa above the submucosal cystic tubules was the eroded pyloric mucosa, and the submucosal tubules appeared to be connected with the dysplastic tubules in the mucosa. In the submucosal tubules, [^{3}H]thymidine-incorporated cells were randomly distributed, and their labeling index was an average of 22.8% (Table 1). The labeling indices of the dysplastic

Table 1

Labeling indices of different kinds of tissue

	Labeling index (%)
Normal pyloric mucosa of control rats	30.6 ± 1.6[a]
Eroded mucosa (at 12th wk)	44.3 ± 4.5[b]
Dysplastic mucosa (at 21st wk)	24.0 ± 3.9[b,c]
Submucosal cystic lesions (at 24th and 27th wk)	22.8 ± 3.6[b,c]
Gastric cancers (at 33rd and 45th wk)	21.2 ± 2.6[b]

[a] Mean ± S.D.
[b] Significantly different from the control, at $p < 0.01$.
[c] Not significantly different from the cancer.

tubules in the mucosa were around 24.0%. In 3 rats killed at the 24th and 27th weeks, small cancers were found to grow in the submucosa. One of the cancers is shown in Fig. 4; its volume was approximately 0.02 cu mm.

From 30 to 33 weeks after MNNG treatment, 4 rats had gastric cancers (Table 2), and the other 4 rats contained no distinct carcinoma but instead had dysplastic, submucosal cystic lesions. Of 16 rats killed after 36 to 45 weeks, 8 rats had carcinomas. All the carcinomas were located at the midpoint of the lesser curvature. Histologically, all the carcinomas were tubular adenocarcinomas; they consisted of large and small, irregularly shaped tubules, lined by relatively basophilic, mucus-depleted cuboidal cells (Fig. 3*D*). The cellular pleomorphism was moderate, but mitotic cells were commonly seen. Of these 8 cancers, 4 were early, in which the neoplastic involvement was confined to the mucosal and submucosal layer. The others were advanced; in 2 rats, serosal invasion was seen. The volumes of the carcinomas were easily measured, because they were a single spherical lesion. The tumor volumes are represented in Table 2. The labeling index of the gastric cancers taken from 33 to 45 weeks after treatment was an average of 21.2% (Table 1). Of 12 rats killed after 36 to 45 weeks, 3 did not have a neoplasm but had healed ulcers at the midpoint of the lesser curvature, and one had no distinct lesion.

Of 8 rats killed after 48 to 51 weeks, 6 had gastric cancers (Table 2) which were adenocarcinomas. Their volumes are shown in Table 2. The growth curve of the gastric carcinomas was obtained by an exponential curve fit on the observed data. The obtained curve was $Y = 0.014 \exp(0.340X)$ ($r^2 = 0.842$), in which r^2 is the coefficient of determination. Hence Y is tumor volume (in cu mm), and X is time (in weeks) after the 24th week (the 24th week was considered to be the starting point (the first week) of tumor growth) (Chart 1). From this, the doubling time of the tumor was estimated to be about 2 weeks. The labeling indices of the carcinomas increased after repeated injections of [^{3}H]thymidine (Table 3). From the randomly selected 3 labeling indices of the gastric cancers after a single injection of [^{3}H]-thymidine (found in the rats killed from 33 to 45 weeks) (Table 3) and those labeling indices of the gastric cancers after 4 and 7 repeated injections at 8-hr intervals, the time required for all the cancer cells to enter S phase was estimated. For this, a linear regression analysis was done. The obtained curve with 95% confidence limits was $Y = [21.6 \pm 5.9 \text{ (S.D.)}] + [1.18 \pm 0.19]X[r^2 = 0.968; F = 213.7]$ (Chart 2). Hence, Y is the labeling index and X is time in hr. From this, it was found that $T_C - T_S$ might elapse in 66.3 hr and T_S might be 18.3 hr. Therefore, the cell cycle time of the cancer cells was considered to be about 84 hr. The labeling indices of the proliferative cell zone in the

Table 2
Incidence and tumor volumes

Time after treatment (wk)	Incidence	Tumor volume (cu mm)
24	1/4	0.0069
27	2/4	0.0182, 0.0853
30	1/4	0.4522
33	3/4	0.9942, 0.4230, 2.3031
36	1/4	0.6013
39	3/4	0.6105, 2.0116, 8.0357
42	2/4	0.3903, 39.5832
45	2/4	20.0464, 100.1532
48	3/4	74.5631, 105.1025, 180.9231
51	3/4	68.2345, 290.4987, 120.6712

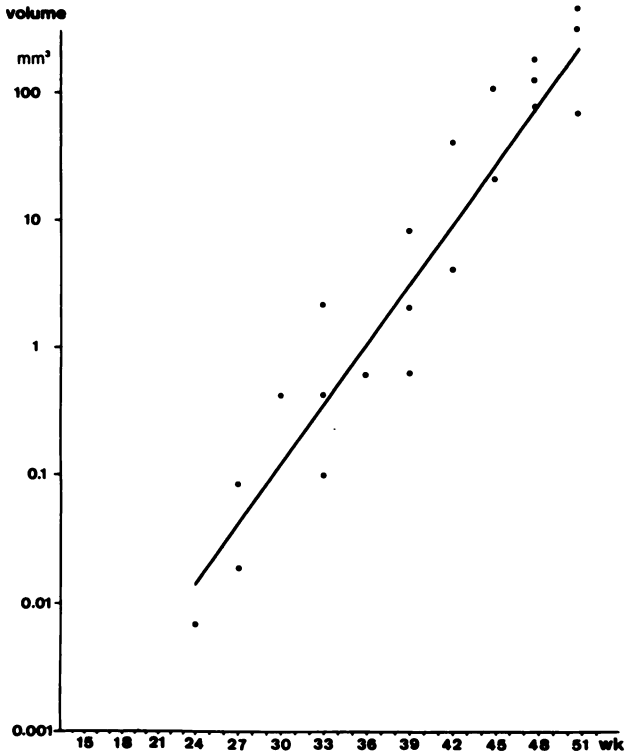

Chart 1. Volumes of gastric cancers found from 24 to 51 weeks after MNNG treatment. Each *point* represents one cancer. The scale of the *ordinate* is logarithmic. The gastric cancer appears to undergo an exponential growth. ——, growth curve, fitted to the observed data.

Table 3
Labeling indices of gastric cancers

	Labeling index (%)
After a single injection of [^{3}H]thymidine	22.6
	20.5
	19.8
After 4 repeated injections of [^{3}H]thymidine	59.2
	49.8
	45.5
After 7 repeated injections of [^{3}H]thymidine	81.9
	78.7
	72.5

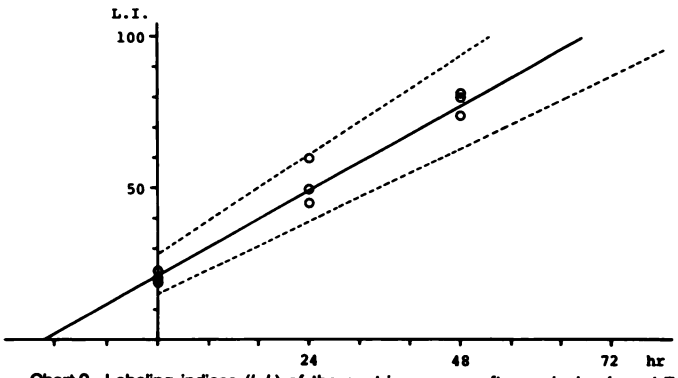

Chart 2. Labeling indices (*L.I.*) of the gastric cancers after a single, 4, and 7 repeated injection(s) of [^{3}H]thymidine (O). ——, fitted curve, obtained by the linear regression analysis of the observed data; - - - -, 95% confidence limits. From the fitted curve, the time required for all the cancer cells to complete their DNA synthesis is estimated to be about 84 hr.

nondiseased antral mucosa became almost 100% by 24 hr of 5 repeated injections of [^{3}H]thymidine.

DISCUSSION

In this study, all benign and malignant lesions induced by MNNG arose at the midpoint of the lesser curvature, confirming the description by Kobori *et al.* (8, 9). It was impossible to provide a clear explanation for this fact, although the area of the gastric mucosa with which MNNG-containing water first comes in contact seems to determine the point where the lesions develop (8). The constant results of this study might depend on an optimal combination of rat strain and MNNG concentration (2, 11); inbred rats seem to provide more reproducible results than do randomly bred rats (8, 9). In this study, the tumor incidence remained very low until the 30th week. This must be due to the difficulty in diagnosing tiny lesions as cancers. After 33 weeks, the tumor incidence was 60.7% (17 of 28).

This study has revealed a sequence of events described by many previous investigators (3, 8, 13). After MNNG treatment, the erosions first arose. The regenerated cells found in the eroded regions of the rats treated with MNNG for less than 18 weeks were not dysplastic but they were similar to those immature cells which were commonly found in the normal stomach. Kobori *et al.* (8) observed ultrastructural and enzyme histochemical characteristics of the regenerated cells in the MNNG-treated rats and found no differences between them and the so-called immature cells in the normal gastric mucosa. Some of the early changes in the gastric mucosa caused by MNNG included the high labeling indices with [^{3}H]thymidine (3). However, the wide distribution of the labeled cells and the high labeling indices were not specific for MNNG treatment; such changes were seen in rats whose stomachs were mechanically injured (7). Until 18 weeks, it was impossible to identify the MNNG-induced dysplastic cells.

In previous studies, many authors described adenomatous changes which preceded the development of gastric carcinomas (8, 9, 13). Some of the previous descriptions of the adenomas included the exophytic growth of the glands toward the luminal surface, and others included the endophytic (downward) growth toward the muscularis mucosae. These changes are brought about by the upward and downward surge in the position and number of DNA-synthesizing cells (3, 12). In this study, exophytic lesions were not seen, and the lesions found before the carcinoma arose were small dysplastic tubules in the submucosa. The glands and/or tubules termed "dysplastic" might be the equivalent of those described by others as adenomatous (10). In these dysplastic tubules, proliferative cells were randomly distributed, and their labeling indices were significantly lower than those of the proliferative cell zone of the control rats (Table 1).

From the 24th week, we could identify gastric carcinomas. The labeling indices of the carcinomas were significantly lower than those of the normal mucosa, but not significantly different from those of the dysplastic mucosa. The low labeling index of a cell population would correspond to an elongation of the cell cycle or a decreased growth fraction (1, 4–6). From the cell kinetic studies of human benign and malignant tumors of the digestive system, it is generally agreed that the DNA-synthetic and cell cycle times elongate as the tumors become malignant (1, 4). As far as the labeling indices with [^{3}H]thymidine were concerned, the forerunner of the carcinomas might be the dysplastic tubules in the eroded mucosa or, by some possibility, the dysplastic tubules might be the carcinomas, whereas they were not identified as malignant because of a lack of morphological manifestation. The cells of the dysplastic tubules must be progenies of the proliferated, immature cells in the eroded mucosa; the repeated contacts to MNNG might have transformed those proliferated cells.

Following repeated injections of [^{3}H]thymidine, the labeling indices of the gastric carcinomas increased. From the linear regression analysis of the data, it could be said that it takes about 84 hr for all the cells to complete their DNA synthesis. This gives a maximum estimate of the cell cycle time, provided that the cells are uniformly distributed in the cell cycle (4, 5). On this assumption, the theoretical doubling time of the gastric carcinomas might be considered to be about 3.5 days. Although the labeled mitosis chase is the best way for the estimation of cell cycle parameters (4), such a study was difficult because it needed many more animals. From the growth curve, the actual doubling time of the carcinomas was shown to be approximately 14 days. The difference between the theoretical and the actual doubling time might reflect a cell loss rate in the carcinomas. If we assume that the doubling time may be about 2 weeks even at the earliest stage of the tumor development, an initial cancer with a volume of about 0.001 cu mm must be found by the time about 7.8 weeks preceding the 24th week [$X = -7.8$ was obtained from $Y = 0.014 \exp(0.340\,X)$ when $Y = 0.001$]. Hence, a volume of 0.001 cu mm may be the minimum size of carcinoma (the diameter of which may be about 130 μm or so) to be detected microscopically. Accordingly, the gastric mucosa of rats treated with MNNG for approximately 16 to 17 weeks must have contained the carcinoma of this size. By 18 weeks after MNNG treatment, however, we found only small dysplastic tubules in the eroded mucosa. These tubules might be the initial stage of the carcinoma in the rat stomach.

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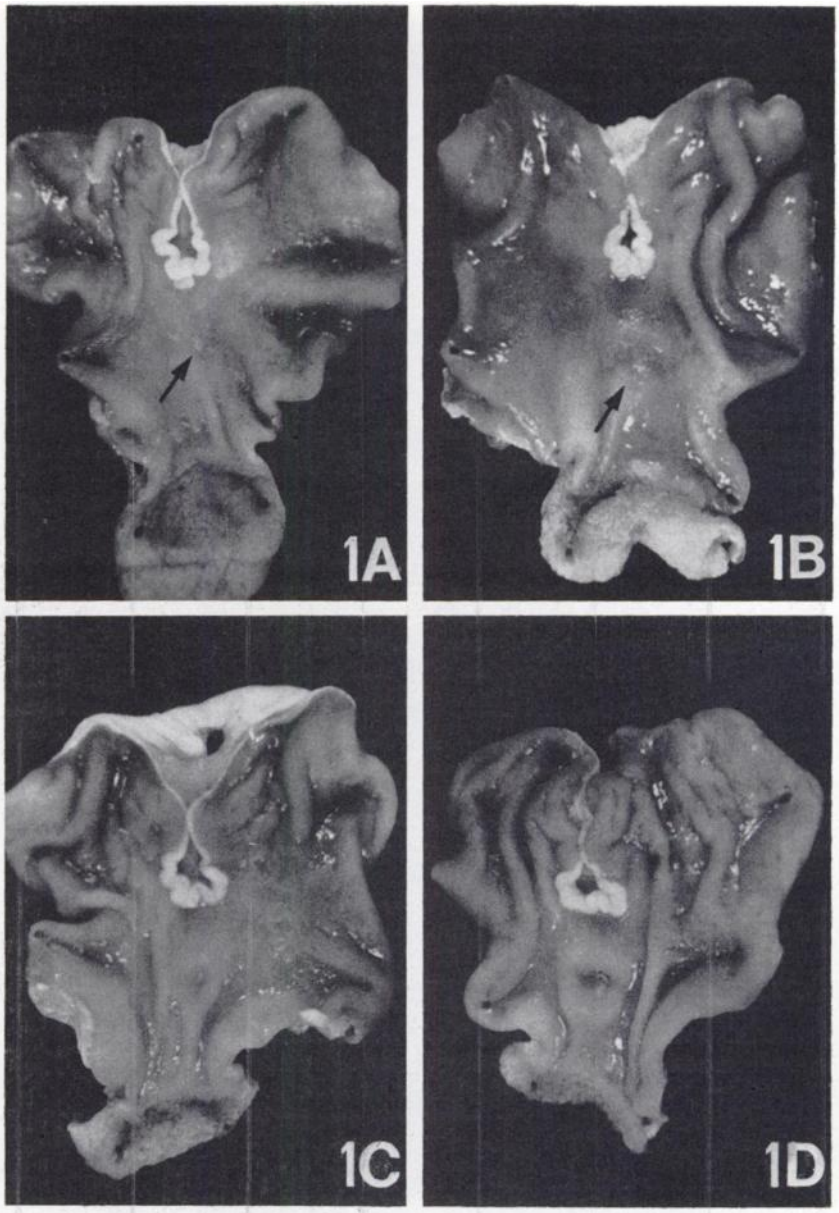


Fig. 1. Macroscopic views of rat stomachs treated with MNNG for 15 (*A*), 27 (*B*), 33 (*C*), and 45 (*D*) weeks; *A*, erosion; *B*, *C*, and *D*, cancers. Note that all the lesions are located at the midpoint of the lesser curvature (*arrows*).

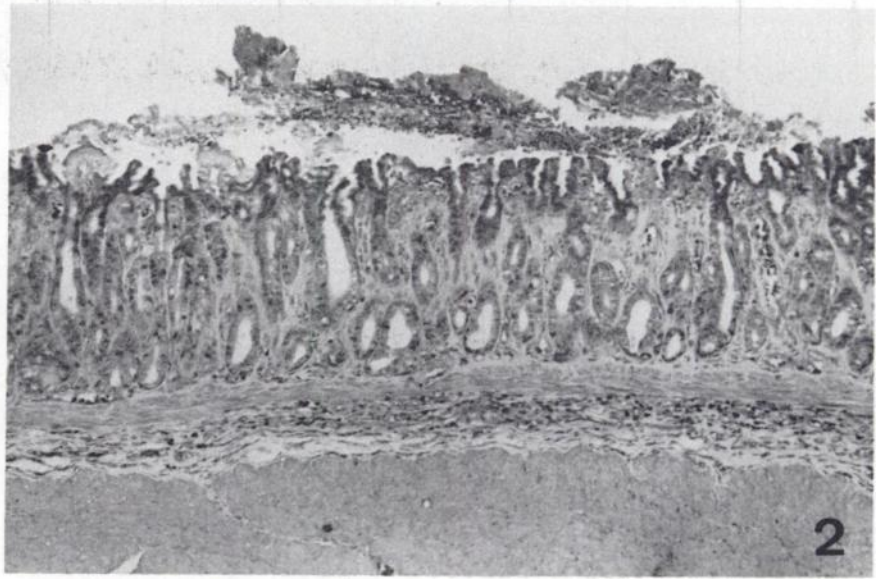
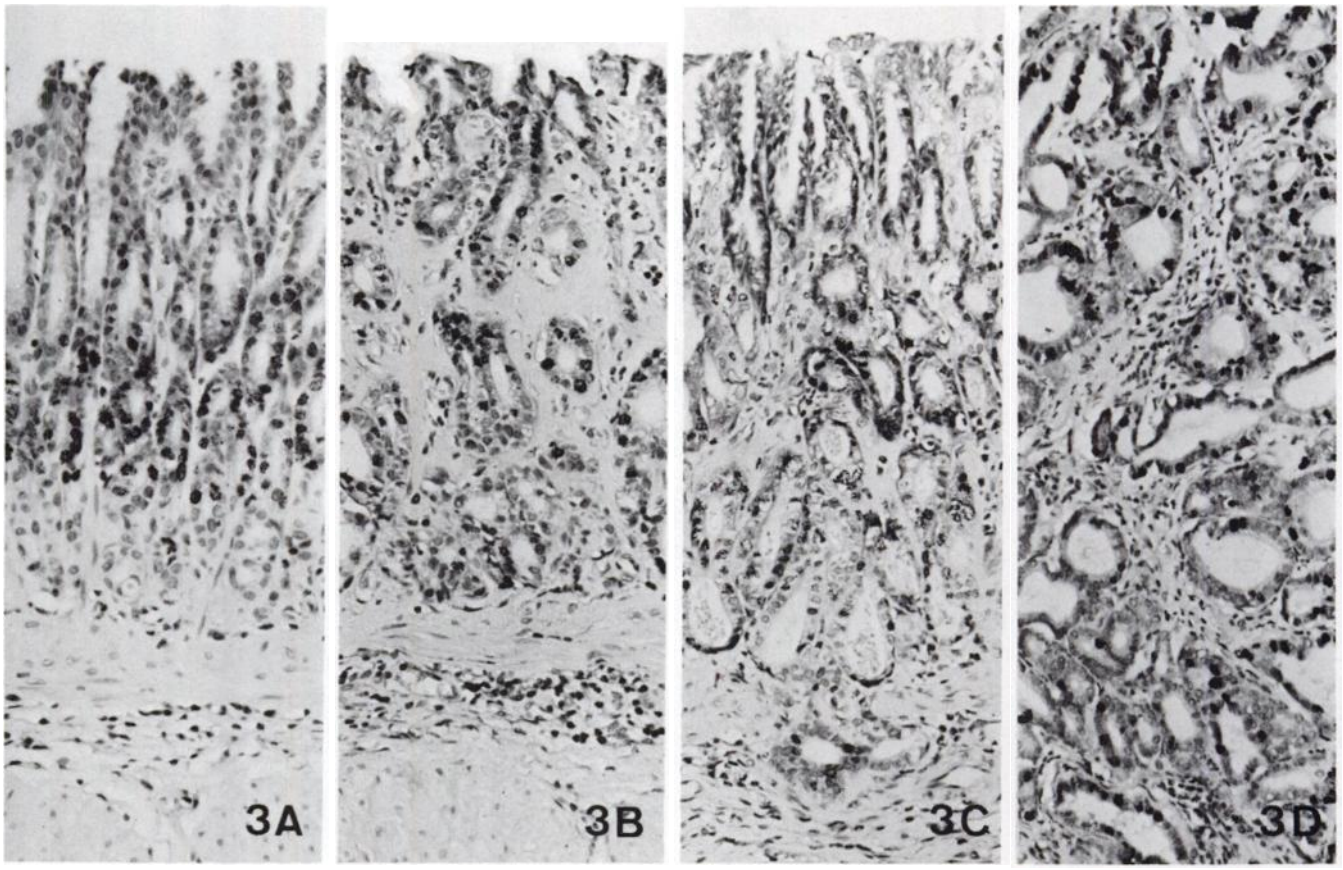


Fig. 2. Histology of erosion of rat stomach treated with MNNG for 15 weeks. Immature regenerative cells are seen. However, structural and cellular atypisms are not detected. H & E, × 120.



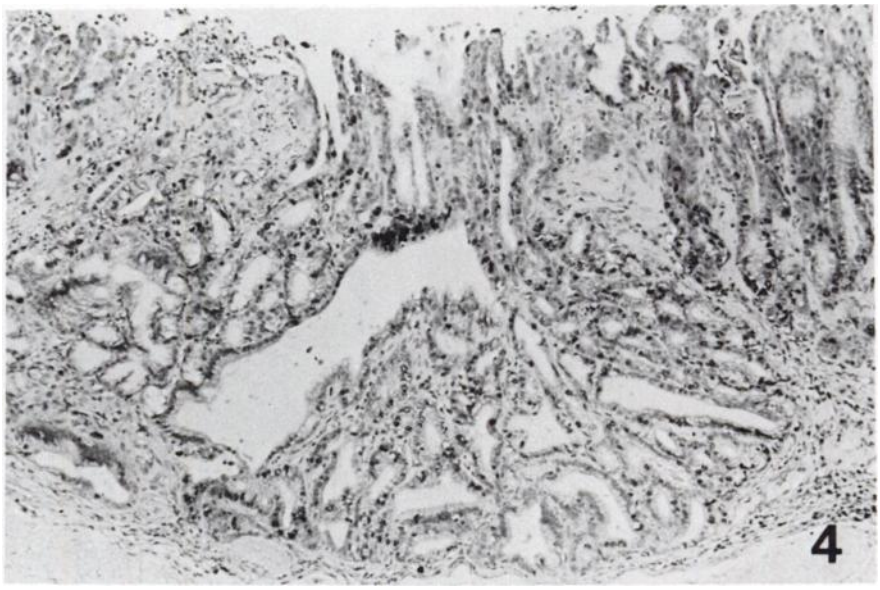


Fig. 3. Autoradiographs of different kinds of tissue after a single injection of [^{3}H]thymidine. *A*, normal pyloric mucosa, in which the labeled cells are confined to the middle third level; *B*, eroded mucosa without dysplasia. The labeled cells are seen widely from the upper to the lower part of the mucosa; *C*, eroded mucosa with dysplasia. The labeled cells are randomly distributed. A small atypical tubule is seen in the submucosa; *D*, adenocarcinoma. The labeled cells are randomly distributed. H & E, × 400.

Fig. 4. Early cancer found at the 27th week. The volume is approximately 0.02 cu mm. H & E, × 210.

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