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Studies on Experimental Colon Tumorigenesis in Rats 2. Cell kinetics of the colon epithelium and its relation to histogenesis of colon tumors*)

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ABSTRACT

1, 2-Dimethylhydrazine (DMH) was administered to a total of 131 Wister Furth substrain (WF/O) rats 20 times weekly at a dose of 20 mg/kg body weight. The tumors were more predominant in the ascending colon (64/148) than in the descending colon (13/148). Examination of the apparently normal colon after the 20th injection of DMH revealed that both the ascending ($P<0.01$) and descending colon epithelia ($P<0.25$) had a higher lebeling index than that of the control epithelia.

Twelve rats which were given a single injection of DMH at a dose of 60 mg/kg of body weight were examined to observe the acute changes in the colon mucosa. This treatment induced a greater injurious effect on the ascending colon than on the descending colon with regard to crypt height and ^{3}H-thymidine labeling index in the nucleus. Recovery from the damage was slower in the ascending colon than in the descenting colon. High iron diamine-Alcian blue staining of the colon epithelia disclosed that sulphomucin was decreased, while sialomucin was increased in the recovery stage in both the ascending and descending colon.

These data indicate that the susceptibility of the colon to the carcinogenic effect of DMH differs by site and that the degree of cellular damage and repair by a single injection of DMH reflects the cell kinetics following a prolonged DMH treatment and ultimate tumor yield.

INTRODUCTION

1, 2-Dimethylhydrazine (DMH) and its related compounds such as azoxymethane (AOM) and methyl-azoxymethanol (MAM) have been widely used to induce experimental colon tumors in rats[9], mice[29] and hamsters[20]. It is known that modification of the administration schedule results in shift of organotropism by the compound. In order to induce colon tumors in rats, DMH should be injected s. c. once a week for about 20 times[14,19]. A smaller does of DMH in drinking water at a shorter interval is effective in inducing liver tumors in rats[4]. It has been reported that a larger dose of DMH and AOM increases the number of small intestinal tumors and descending colon tumors in some strains of rat[30,32]. The organotropism of these chemicals to the colon has been accounted for partly by excretion of glucuronated metabolites in the bile and reactivation of the carcinogen by intestinal flora[22,31]. However, in experiments using germ free animals[22] or making partially isolated colon loops[25] it has been observed that a high susceptibility to these carcinogens exists in the colon epithelium *per se*.

In our previous study a marked strain dif-

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ference in incidence, location, and type of tumors was observed between Wistar Furth substrain rats (WF/O) and Long Evans rats[18]. By the administration of DMH some rat strains develop more tumors in the right colon (random Wistar[13]), while other rat strains in the left colon (BD IX[16], Wistar Porton[27], Long Evans[18]). Our WF/O rats belong to the former type with tumors predominantly developing in the right colon. Experimental studies were made on the histogenesis of rat colon tumors induced by DMH from the viewpoint of cell kinetics and mucin histochemistry.

MATERIALS AND METHODS

Chronic experiment and acute experiment were conducted in this study.

Experiment I (chronic experiment)

Animals A total of 131 Wistar Furth substrain (WF/O) rats were used in the chronic experiment. They were produced in our laboratory from the parents of WF/O rats given by the courtesy of Dr. M. Miyamoto, Osaka University, 1978. They were bred by brother-sister mating, housed in plastic cages 3 or 4 per cage, in an air-conditioned room maintained at 24±2°, and given commercial pelleted diet (Oriental MF) and water *ad libitum*.

Carcinogen 1, 2-Dimethylhydrazine dihydrochloride (DMH, Nakarai Chemicals, Ltd., Kyoto) adjusted to pH 6.5 with $NaHCO_3$ was injected s.c. on the back weekly for 20 weeks at a dose of 20 mg/kg of body weight.

Experimental Plan Animals were divided into 4 groups. In group I composed of 20 males and 20 females DMH was administered from the age of 5 weeks and in group II composed of 13 males and 12 females, DMH treatment was started at the age of 10 weeks. Group III having 20 males and 19 females and group IV having 12 males and 15 females were the matched controls of group I and group II, respectively. Animals were weighed once a week and sacrificed 30 to 45 weeks after the initial treatment of DMH.

Preparation of Specimens of the Gastrointestinal Tract Complete autopsy was performed on all the animals and tissue specimens were obtained from the representative sites of the gastrointestinal tract including both tumorous and non-tumorous areas. They were fixed in 10% neutral formaldehyde, embedded in paraffin, cut 4 μm in thickness and stained with hematoxylin and eosin (HE). Selected tissues were stained by periodic acid-Schiff (PAS) method and high iron diamine-Alcian blue (pH 2.5) (HID-AB) method[26] to clarify the nature of mucin in the mucosa.

Autoradiography A total of 44 rats belonging groups II and IV were used to determine changes in DNA-synthetic activity of crypt cells and tumor cells. These rats were given an i.p. injection of tritiated thymidine (^{3}H-TdR, specific activity 2.0 Ci/mmol; Japan Radioisotope Association) dissolved in 0.9% NaCl solution at a dose of 0.5 μCi/g of body weight one hour prior to sacrifice. Deparaffinized sections were dipped in autoradiographic emulsion (NR M2, Konishiroku Photo Ind. Co., Tokyo) at 40°, exposed for 4 weeks at 4° in a dark room, and then stained with hematoxylin and eosin. Cells about 500 in number were counted in the complete crypts with precaution taken to exclude crypts adjacent to the tumor. A cell with 4 or more grains on the nucleus was recorded as positive.

Experiment II (acute experiment)

Animals Twelve 10-week-old female Wistar Furth substrain rats bred as in Experiment I were used.

Experimental Plan DMH solution prepared as in Experiment I was administered to 10 rats at a dose of 60 mg/kg of body weight. Two animals each were sacrificed on day 1, 2, 3, 5 and 7 after s.c. injection of DMH. Two rats were used as untreated controls (day 0).

One hour prior to sacrifice, i.p. injection of ^{3}H-TdR (specific activity 27 Ci/mmol; Japan Radioisotope Association) was made at a dose of 1.0 μCi/g of body weight. Tissue specimens and autoradiographic sections were prepared as in Experiment I. Ten complete crypts of both the ascending and descending colon were examined in each animal.

Statistical Analyses χ^2 and "t" tests were used to determine the significance of difference in the indices.

RESULTS

Experiment I

Incidence and Distribution of Intestinal Tumors The incidence of colon tumors in group I was 100% in male rats and 80% in female rats. The incidence was lower in group II,

Table 1. Incidence and Number of 1, 2-Dimethylhydrazine-Induced Colon Tumors in WF/O Rats

Experimental group	Age at start	Sex	Treatment	No. of rats	No. of rats with colon tumor (%)	Total No. of colon tumors	No. of colon tumors per rat
Group I	5W	M	DMH	20	20(100)	51	2.6
	5W	F	DMH	20	16(80)	26	1.6
Group II	10W	M	DMH	13	11(85)	25	2.3
	10W	F	DMH	12	7(58)	17	2.4
Group III	5W	M	Control	20	0	0	0
	5W	F	Control	19	0	0	0
Group IV	10W	M	Control	12	0	0	0
	10W	F	Control	15	0	0	0

Table 2. Histological Types of Intestinal Tumors in Relation to Site

Site	Total No. of intestinal tumors[a)]	Histological type			
		Adenoma (%)	Adenocarcinoma well & mod. (%) differentiated	Adenocarcinoma poorly (%) differentiated	Not examined (%)
Cecum & ascending colon	64	11(17)	35(55)	17(27)	1 (1)
Pars flexura lienalis	42	7(17)	30(71)	5(12)	0
Descending colon	13	2(15)	10(77)	1(8)	0
Small intestine	29	16	7	6	0
Total	148	36	82	29	1

a) Tumors in male and female rats are combined.

being 85% in male rats and 58% in female rats. No intestinal tumors were observed in the control rats (groups III & IV) (Table 1.).

As the distribution pattern of colon tumors in group I was quite similar to that of group II, these two groups were combined (Table 2). A total of 148 intestinal tumors, that is, 119 colon tumors and 29 small intestinal tumors, were observed. In order to examine the distribution of tumors by sites, the colon was divided arbitrarily into three parts, i. e. the ascending colon (including the cecum), pars flexura lienalis, and descending colon. The number of tumors in these sites was 64, 42 and 13, respectively. Tumors were more prevalent in the ascending colon where poorly differentiated adenocarcinomas were found more frequently than the other sites of the colon.

Proliferative Zone in the Crypt of the Colon Epithelium The labeled cells in the ascending colon, were prevalent in the middle third of the crypt with none in the bottom. On the contrary, in the descending colon the labeled cells were numerous in the lower third of the crypt (Figs. 4, 5). After DMH treatment the proliferative zone was extended widely and the crypt became higher than that in the control (Figs. 6, 7).

The Labeling Index in the Non-Tumorous Mucosa of the Ascending and Descending Colon Table 3 shows the results obtained by

Table 3. Lebeling Index in Non-tumorous Mucosa

Treatment	Ascending colon %±SEM	Descending colon %±SEM
DMH	16.3±1.8(9)[a)b)]	13.1±1.9(8)[c)]
Control	9.8±0.6(19)	9.6±0.6(18)

a) Figures in parentheses indicate the number of animals examined.
b) $P<0.01$, DMH versus control.
c) $P<0.25$, DMH versus control.

comparing the number of ^{3}H-TdR-labeled cells to the total number of cells (labeling index). After 20 injections of DMH, crypts appearing normal in the ascending colon had higher labeling index (16.3) than the control (9.8) ($p<0.01$). The descending colon also showed the same tendency (13.1 vs. 9.6) ($p<0.25$).

Labeling and Mitotic Indices in Various Types of the Colon Tumors Tumors were classified into three histological types, i.e. adenoma, well and moderately differentiated, and poorly differentiated adenocarcinoma. The ^{3}H-TdR uptake was more pronounced in benign adenomas (the mean value is 29.4), followed by the well and moderately differentiated adenocarcinomas (23.0) and the poorly differentiated adenocarcinomas (14.6) (Table 4). The labeling index of poorly differentiated adenocarcinoma was almost identical to that in the non-tumorous colon mucosa treated with DMH (c.f. Table 3). Mitotic index was also higher in adenoma than in any other type.

Experiment II

Staining Property of the Colon Epithelium after a Single Injection of DMH The staining characteristics of the mucosa after a single DMH treatment are shown in Table 5. Neither HE nor PAS but HID-AB staining could make a definite distinction between the ascending colon and descending colon. In the ascending colon of the control rats, HID positive goblet cells (stained black or brown for sulfomucin) were seen only in the upper third of the crypt, but AB positive cells (stained blue for sialomucin) were dominant in the middle and lower third of the crypt. In the descending colon of the control rats, HID positive cells were prevalent in the crypt and few AB positive cells were seen.

In the ascending colon from day 3 to 5, HID positive cells disappeared in the crypt and all the goblet cells were stained blue. HID positive cells began to appear on day 7. In the crypts of the descending colon HID positive cells decreased and AB positive cells increased from

Table 4. Labeling and Mitotic Index in Colon Tumors

Adenoma		Adenocarcinoma			
		well & moderately differentiated		poorly differentiated	
^{3}H-thymidine labeled cells (%)	Mitotic cells (%)	^{3}H-thymidine labeled cells (%)	Mitotic cells (%)	^{3}H-thymidine labeled cells (%)	Mitotic cells (%)
29.4± 3.0	1.97± 0.3	23.0± 1.6	1.45± 0.2	16.1± 3.3	1.04± 0.1
(3)	(3)	(11)	(11)	(9)	(9)

Figures in parentheses indicate the number of tumors examined.
Values are mean±SEM.

Table 5. Changes in Staining Characteristics after a Single Injection of DMH

Site of the colon	Days after a single injection of DMH											
	control		1		2		3		5		7	
	HID[a]	AB[b]	HID	AB	HID	AB	HID	AB	HID	AB	HID	AB
Ascending colon	+	++~+++	±	+~++	±	++	−	++	−	+++	±	++~+++
Descending colon	+++	−	++~+++	−	+++	±	++	±	++	+	++	+

a) HID means high iron diamine which stains sulfomucin black or brown.
b) AB means Alcian blue which stains sialomucin blue.
+++ markedly positive
++ moderately positive
+ slightly positive
− negative

day 2 to 7.

Changes in Total Number of Cells per Crypt The number of cells per crypt continued to decrease until day 2 after treatment in both the ascending and descending colon (Fig. 1). Then

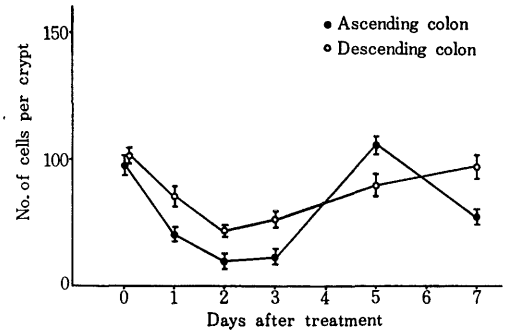

Fig. 1. Total number of cells per crypt after a single administration of 60 mg/kg of DMH in WF/O rats. Values are mean±SEM.

the crypt cells began to increase gradually in the descending colon and returned to the control level on day 7 (Figs. 8–15). On the other hand, in the ascending colon the number of cells abruptly increased day 5 and then dropped on day 7. The cell loss was more severe in the ascending colon than in the descending colon during the initial phase (up to day 3).

Labeling and Mitotic Indices of the Colon Epithelium after A Single Injection of DMH The labeling index of colon epithelium decreased as early as day 1 more markedly in the ascending colon (Fig. 2). On day 2 the indices recovered to the control level and overshot on day 3. On day 5 the labeling index returned to the control level in the descending colon but it continued to maintain a high level in the ascending colon and recovered to the control level on day 7.

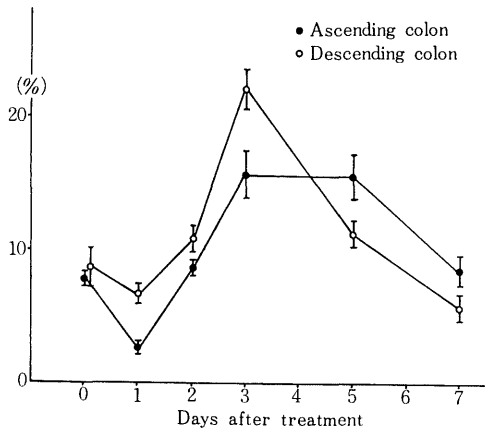

Fig. 2. Labeling index of colon after a single administration of 60 mg/kg of DMH in WF/O rats. Values are mean±SEM.

In the meantime, mitotic index decreased drastically on day 1 in both the ascending and descending colon. Thereafter in the ascending colon the indices returned to the control level from day 2 (Fig. 3), while in the descending colon they overshot trasiently on day 3 and began to decline on day 5 to exceed the control value on day 7.

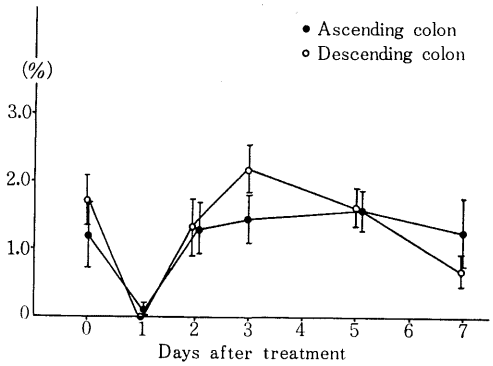

Fig. 3. Mitotic index of colon after a single administration of 60 mg/kg of DMH in WF/O rats. Values are mean±SEM.

DISCUSSION

The mechanism of the difference in susceptibility of the various sites of the bowel to DMH carcinogenesis has not been well understood. Maskens found that acute nucleotoxic reaction was more severe in the transverse colon, the site of highest tumor yield in BD IX rats[17]. Sunters et al. stated that after chronic DMH treatment the number of cells in the crypt rose in both the transverse colon and the descending colon where tumors were prevalent[27].

In the present experiments, colon tumors developed more frequently in the ascending colon (Exp. I), where the total number of cells per crypt was more markedly decreased and a high level of labeling index was observed for a longer period than in the descending colon following a single injection of DMH (Exp. II). These findings suggest that there are some differences in susceptibility to DMH between

the ascending colon and descending colon and that there is some correlation between the degree of initial damage and the subsequent tumor yield. The potential factors which may contribute to the difference in susceptibility of the various sites of the bowel are the amount of DMH-metabolizing enzyme, the number of target cells, and the repair capacity of the damaged cells together with the frequency of DNA replication. As for DMH-metabolizing enzymes, the ascending colon of the mouse lacks the enzymes which can convert DMH to ultimate carcinogen, and tumors develop only on the descending colon which is abundant in specific enzymes[1]. When the number of target cells in the cecum is increased by surgical pretreatment, the number of tumors becomes also increased at that site[21].

DMH is metabolized[5] and methylates nucleic acid in the colon mucosa as well as in other tissues (liver and kidney[11]). 7-Methylguanine is reportedly the major product[10]. However, when the importance of hydrogen binding of nucleotide pairs is taken into consideration, methylation of other positions of purine and pyrimidine seems to be more important[8,15]. Goth has shown that N-ethyl-N-nitrosourea (ENU), which is carcinogenic for the developing nervous system, ethylates DNA of the liver cells as well as that of brain cells and produces 7-ethylguanine and O-6-ethylguanine *in vivo*. However, repair of O-6-ethylguanine is slower in the brain than in the liver, leading to selective tumorigenesis in the brain[9]. There may be such an enzymatic difference in repair between the ascending colon and descending colon.

In the present study, HID-AB staining has revealed that the distribution ratio of two kinds of mucins is different between two different parts of the colon and that during the course of regeneration sulfomucin tends to decrease, while sialomucin increases in both parts of the colon. These deta are compatible with the observation in mice that crypts containing sialomucin are rather immature in nature[28]. The difference in the amount of mucosal carbohydrate and enzymes involved in glycoprotein metabolism between the ascending and descending colon[6] was indirectly confirmed by our findings obtained in HID-AB staining study.

It has been noted that after several administrations of DMH the labeling index rises significantly in the apparently normal mucosa of treated mice[2,24]. In the present study, both the ascending colon and descending colon had higher labeling indices than that of the control after chronic DMH treatment with a statistically significant difference only in the ascending colon. This indicates that DNA synthesizing capability in the normal appearing mucosa is much higher than that in the control. These results suggest that the severe damage of the epithelium and the long-pending elevated labeling index in the ascending colon ultimately lead to persistently elevated DNA synthesis in the mucosa of the ascending colon as a result of the cumulative effect of repeated DMH treatment.

The crypt having a higher labeling of ^{3}H-TdR may contain larger cell population of "initiated cells" capable of responding to "tumor promoters" such as dietary fat and protein, bile acid[23], and bacteria[22], and finally develops into multiple tumors at that area. The two-step theory in tumorigenesis is well established for skin tumors and hormone-related tumors[7]. It is reported that SENCAR mice whose epidermal cells have a higher ^{3}H-TdR labeling index than BALB/c mice are more sensitive to initiators and promoters in skin tumorigenesis[12]. It is reasonable to assume that the mucosa with higher labeling index in the consequence yields multiple tumors. However, once the tumor develops, the labeling index of the tumor has little correlation to malignancy; the labeling indices of adenocarcinomas were lower than that of adenoma in this study. In conclusion, the present study has shown that there is a difference in susceptibility to the carcinogenic effect of DMH by site of the colon and that the histological and cell kinetic changes in the mucosa following a single administration of DMH is predictive of the chronic changes of epithelial cell kinetics after a prolonged DMH treatment and of subsequent tumor productivity.

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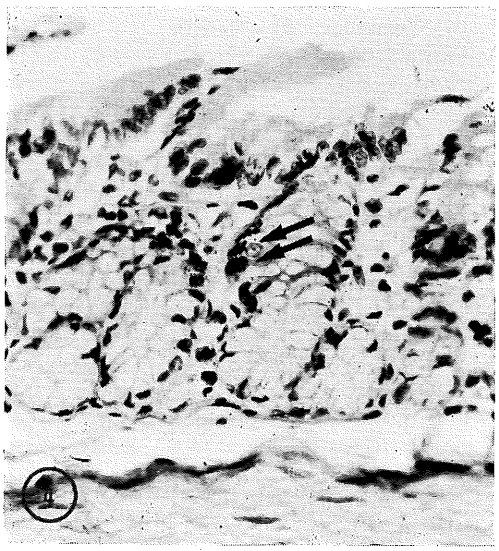

Fig. 4. Ascending colon of the control rat. Forty-five weeks of age. DNA-synthetizing cells are located in the middle one-third of the crypt (arrows).

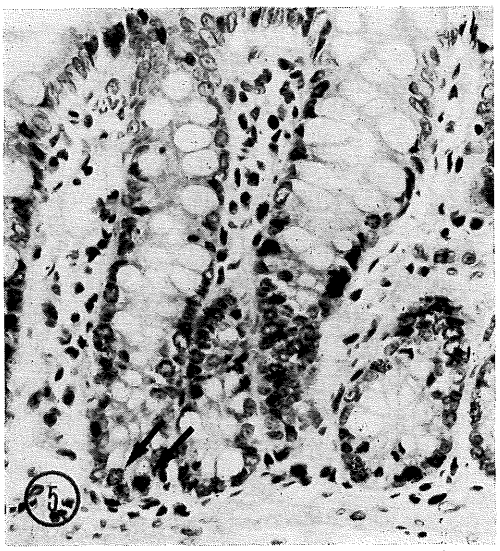

Fig. 5. Descending colon of the control rat. Forty-five weeks of age. DNA-synthetizing cells are located in the lower one-third of the crypt (arrows).

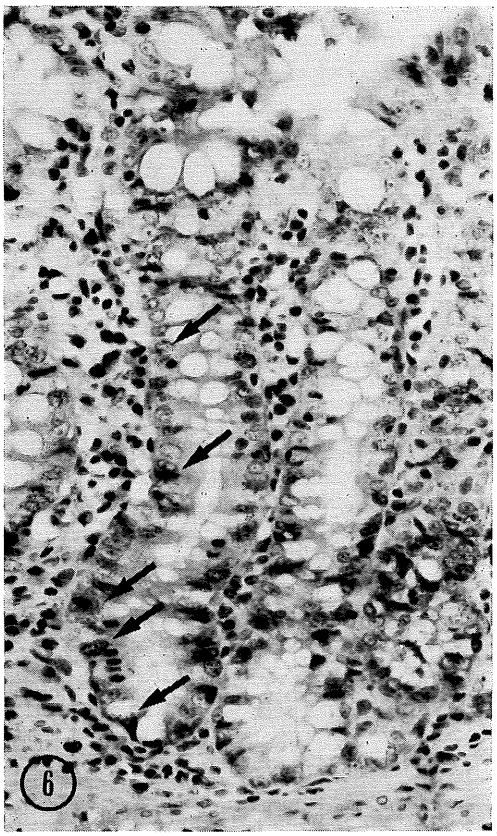

Fig. 6. Ascending colon. DNA-synthetizing cells are increased in number and found in the lower two-thirds of the crypt (arrows). Number of cells per crypt is also increased.

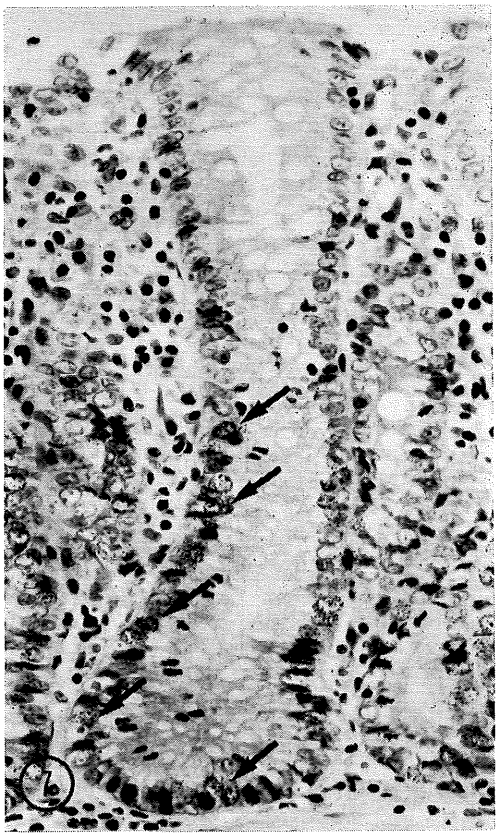

Fig. 7. Descending colon. DNA-synthetizing cells are increased in number and observed in the lower two-thirds of the crypt (arrows). Number of cells per crypt is also increased.

Fig. 4-7. Microautoradiographs of the ascending and descending colon of WF/O rats, treated with 20 times weekly injection of 20 mg/kg of DMH. Hematoxylin and eosin staining. × 200

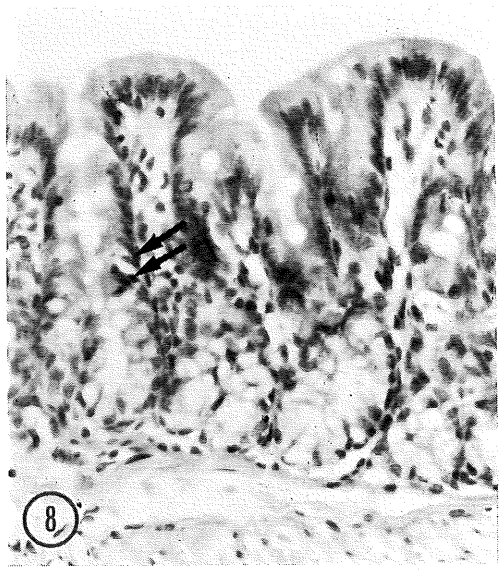

Fig. 8. Ascending colon of the control rat. Ten weeks of age. ^{3}H-TdR-labeled cells are in the middle one-third of the crypt (arrows).

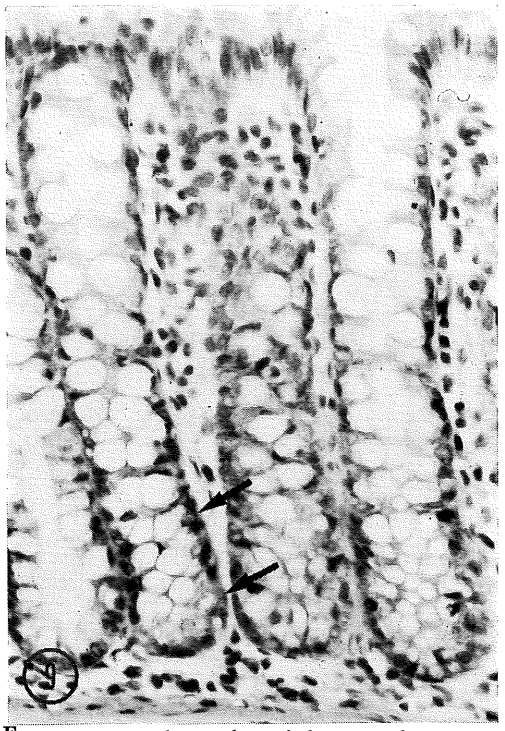

Fig. 9. Descending colon of the control rat. Ten weeks of age. ^{3}H-TdR-labeled cells are in the lower one-third of the crypt (arrows).

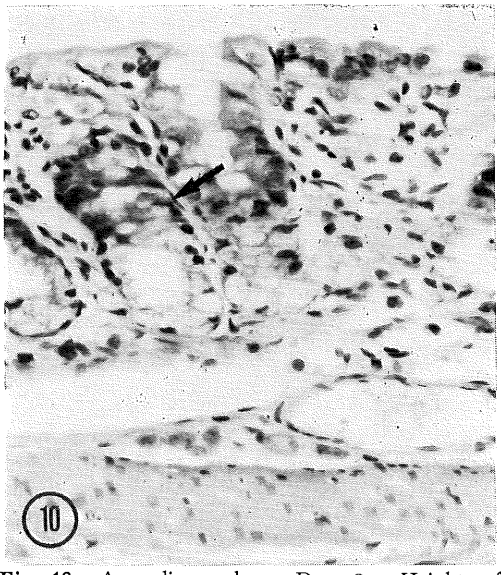

Fig. 10. Ascending colon. Day 2. Height of crypts is shortened remarkably. A few DNA-synthetizing cells are observed (arrow).

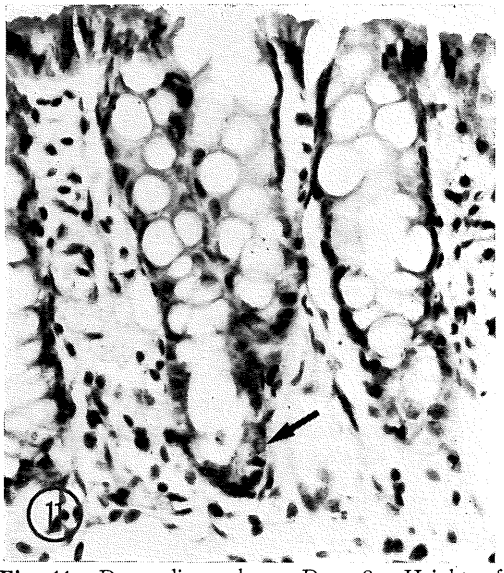

Fig. 11. Descending colon. Day 2. Height of crypts is shortened moderately. A few DNA-synthetizing cells are observed (arrow).

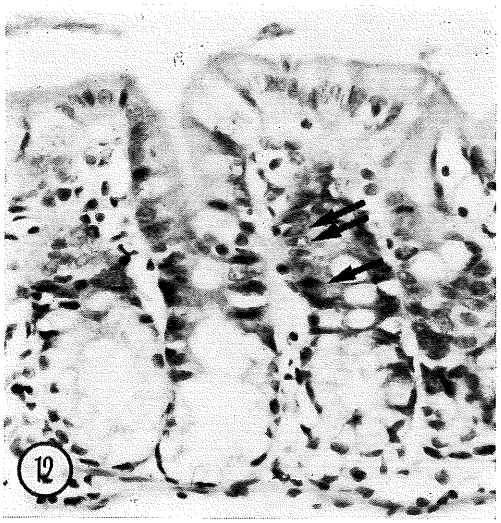

Fig. 12. Ascending colon. Day 3. Height of crypts is still lower than control. Many DNA-synthetizing cells are observed in the middle one-third of the crypt (arrows).

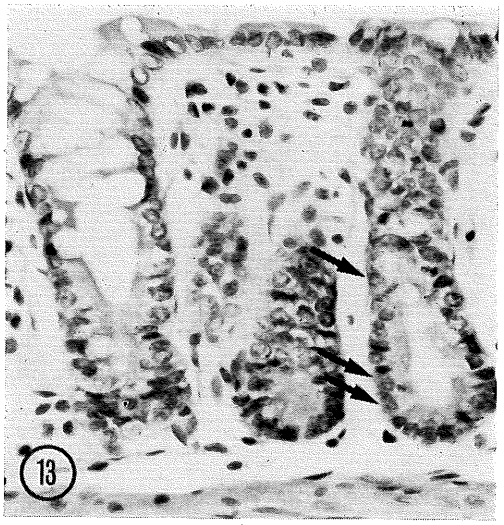

Fig. 13. Descending colon. Day 3. Height of crypt is still lower than control but many DNA-synthetizing cells are observed in the lower half of the crypt (arrows).

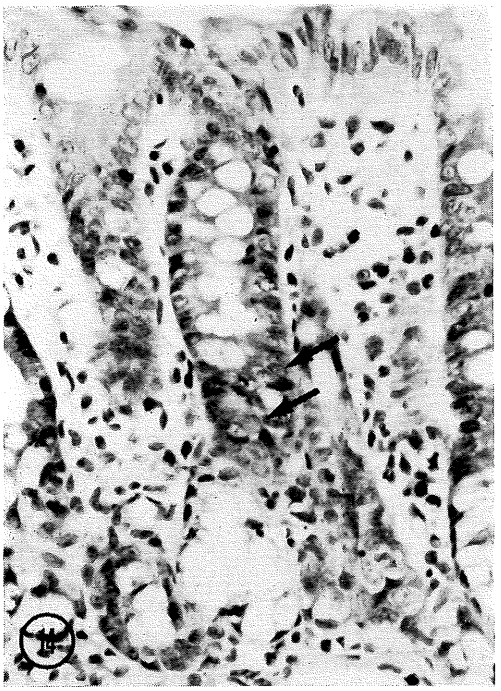

Fig. 14. Ascending colon. Day 5. Crypts are elongated than control. DNA-synthetizing cells are in the middle one-third of the crypt (arrows).

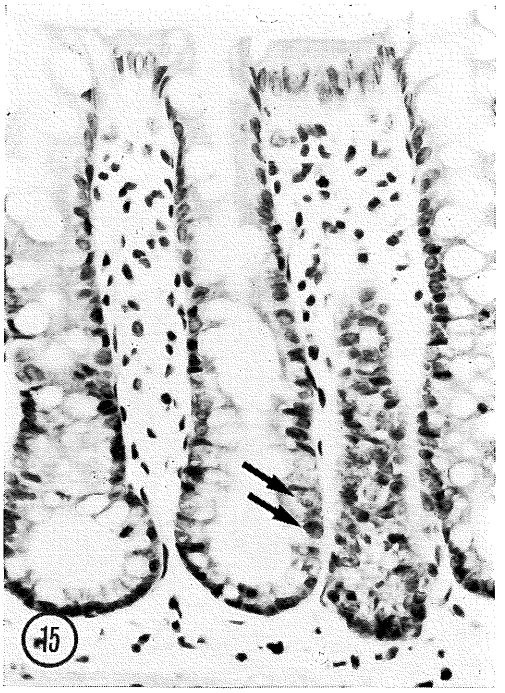

Fig. 15. Descending colon. Day 5. Height of crypt and number of labeled cells (arrows) are returned to control values.

Fig. 8-15. Microautoradiographs of the ascending and descending colon of WF/O rats treated with a single s. c. injection of 60 mg/kg of DMH. Hematoxylin and eosin staining. × 200