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Growth Stimulation of Rat Colonic Cells in Organ Culture with *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine (41557)

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Abstract. The effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) on rat colonic mucosa were investigated in organ culture. Distal colonic mucosa separated from the muscle layers was cultured on a substrate of human fibrin foam. Exposure of colonic organ cultures to 0.1, 1.0, and 10.0 μg MNNG/ml of medium on a multiple discontinuous basis, e.g., every third day for 3 hr, produced significant carcinogen effects. [^{3}H]Thymidine was incorporated throughout crypts and in surface epithelium of carcinogen-treated explants. Outgrowth of epithelioid cells into the fibrin foam matrix was observed in all treated explants 9 days after initial MNNG exposure. Control untreated cultures showed limited outgrowth. After 15 and 21 days in culture, epithelioid outgrowth was still observed in 1.0 μg MNNG/ml-treated cultures.

Explants of rat colonic mucosa in organ culture respond to carcinogens (1, 2), making this model useful for studies of colon carcinogenesis. Administered intrarectally, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a direct-acting alkylating agent (3), produces a high incidence of colon tumors (4, 5). Because MNNG exerts a direct effect without intervening metabolism, this chemical is excellent for studying effects of carcinogens on target colonic epithelium in organ culture.

We recently reported successful culture of rat distal colonic mucosa for 3 to 4 wk (6), an interval suitable for conducting *in vitro* carcinogenesis investigations. The present study documents the short-term effects of MNNG on cultured rat colonic mucosa using human fibrin foam as the matrix.

Materials and Methods. Organ cultures were prepared from the distal colon of 7- to 8-week-old male Fischer 344 rats (Charles River Breeding Labs., Wilmington, Mass.) by previously described methods (6). Colonic explants were initially cultured on human fibrin foam for 18 hr in carcinogen-free supplemented Waymouth's MB 752/1 medium (6) with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, N.Y.) at 37°C in 95% O_2–5% CO_2. MNNG (Aldrich Chemical Co., Milwaukee, Wisc.) was dissolved in 0.85% NaCl and diluted in the culture medium to final concentrations of 0.1, 1.0, and 10 μg MNNG/ml (6.8×10^{-7} *M*, 6.8×10^{-6} *M*, and 6.8×10^{-5} *M*, respectively) and colonic explants were exposed discontinuously (every third day for 3 hr). Control explants were incubated in medium without MNNG. At 3, 9, 15, and 21 days after initiation of MNNG treatment, three to six explants per dose were incubated at 37°C in 95% O_2 for 4 hr in serum-free supplemented Waymouth's MB 752/1 medium containing 25 μCi [methyl-^{3}H]thymidine/ml (sp act 20 Ci/mmol, Research Products International Corp., Elk Grove Village, Ill.). The cultures were then transferred to serum-free supplemented Waymouth's MB 752/1 medium containing a ten-fold greater concentration of nonradioactive thymidine (Sigma Chemical Co., St. Louis, Mo.) and incubated at 37°C in air–5% CO_2 for 30 min. Cultures were fixed *in situ* on the fibrin foam and sectioned at right angles to the foam. For light microscopy, 5-μm paraffin sections were prepared and stained with hematoxylin and eosin (H & E). Survival rating of epithelial and stromal structures was determined by criteria previously described (6). In addition, a detailed assessment of epithelial maintenance was made according to criteria described by Defries (7). For autoradiography, deparaffinized sections were dipped in Kodak NTB2 nuclear track emulsion (1:2 distilled water), dried, and exposed for 1 week at 4°C. The sections were processed in Kodak D-19 developer for 3 min, then stained with H & E.

Results. Control cultures maintained in carcinogen-free medium showed histological features of cultured colonic mucosa previously described (6). Explants retained a variable number of glandular crypts with normal

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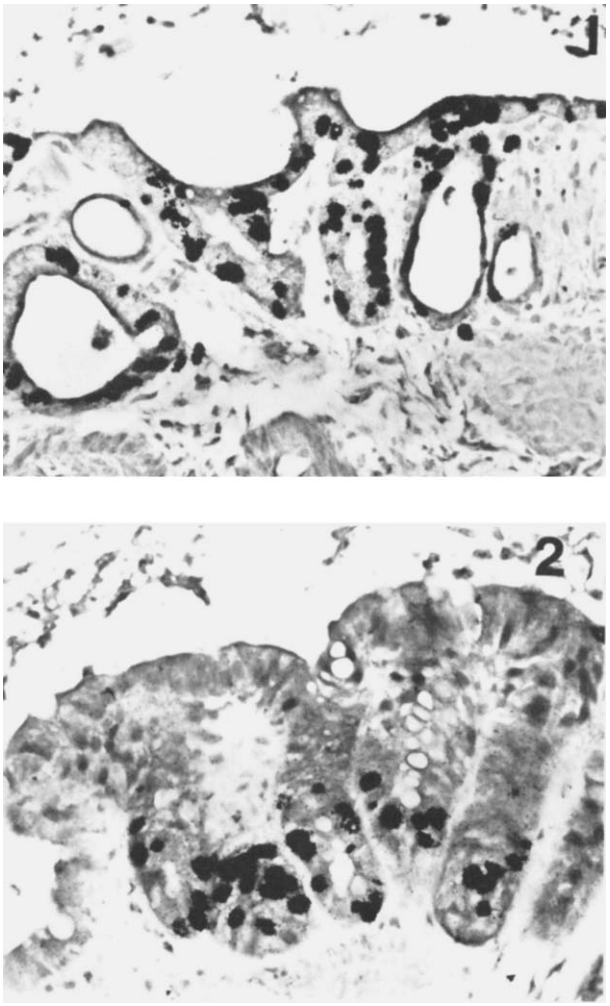


FIG. 1. Autoradiograph of [^{3}H]thymidine incorporation into MNNG-treated colonic mucosa. The explant was exposed discontinuously to 1.0 μg MNNG/ml (three 3-hr exposures) and examined 9 days after the initial exposure. H & E. ×200.

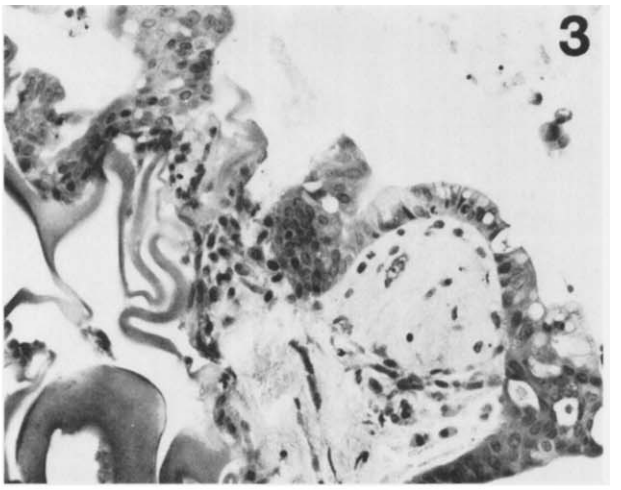
FIG. 2. Autoradiograph of [^{3}H]thymidine incorporation into carcinogen-free medium control after 9 days in organ culture. H & E. ×240.

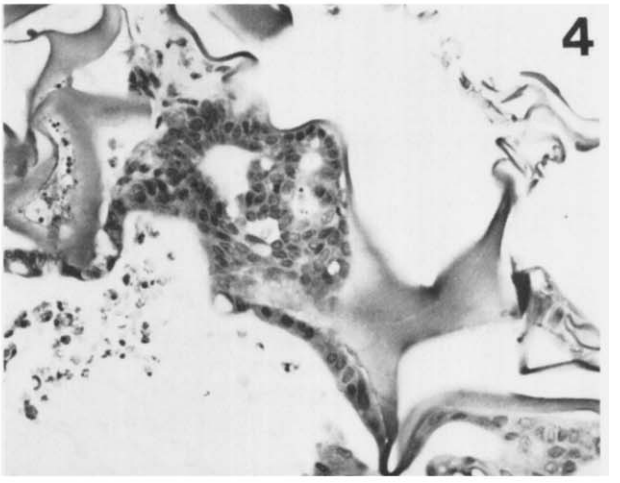
FIGS. 3 AND 4. Outgrowth of epithelioid cells from explants treated with 1.0 μg MNNG/ml on a discontinuous schedule for 9 days (three 3-hr exposures). H & E. ×200.

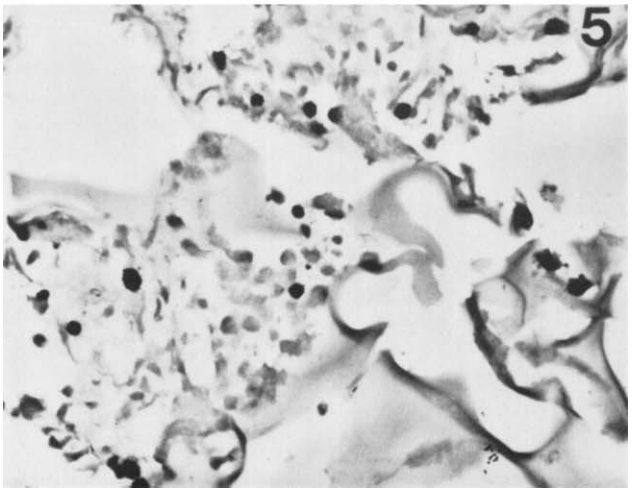
FIG. 5. Autoradiograph of [^{3}H]thymidine incorporation into epithelioid outgrowth from explants exposed on a discontinuous schedule to 1.0 μg MNNG/ml, 9 days after initial exposure (three 3-hr exposures). H & E. ×200.

columnar epithelium for 14 to 21 days in culture. Crypts were of various length; some were normal and others short and wide. Cellular outgrowth from the edge of the explant was limited.

The histological appearance of explants exposed discontinuously to 0.1, 1.0, and 10 μg MNNG/ml showed moderate changes when compared to untreated control explants 9 days after the initial MNNG exposure (Table I).







Figs. 3, 4, 5 (*Continued*)

TABLE I. SURVIVAL OF COLONIC MUCOSAL ORGAN CULTURES DISCONTINUOUSLY EXPOSED TO VARIOUS CONCENTRATIONS OF MNNG

Days after initial MNNG exposure	No. of 3-hr exposures	Conc. MNNG (μg/ml)	No. of explants showing: Mucosal survival[a] I	II	III	Epithelial survival[b] A	B	C	D	Epithelioid cell growth into foam
3	1	0		6			6			No
		0.1	1	2		2	1			No
		1.0		3			3			No
		10.0		2	2		1	3		No
9	3	0		4	1		4		1	No
		0.1		3	1		3	1		Yes
		1.0		2	2		2		2	Yes
		10.0		3	1		3	1		Yes
15	5	0		4			3	1		No
		0.1			3				3	No
		1.0			4			1	3	Yes
		10.0			5				5	No
21	7	0		2	2		1	3		No
		0.1			5				5	No
		1.0			5				5	Yes
		10.0			6				6	No

[a] Mucosal survival: I—no degeneration, II—moderate degeneration, III—marked degeneration.
[b] Epithelial survival: A—fully differentiated surface epithelium with glandular crypts; B—explants covered with layer of well-preserved epithelial cells with modified crypts; C—few undifferentiated cells, regenerating in unorganized form; D—no epithelial survival.

The colonic crypts were lined with equal numbers of mucus-producing cells and epithelial cells. The length of the colonic crypts in the MNNG-treated explants was reduced compared to untreated control tissue, and focal dilation was observed. The lamina propria in the carcinogen-treated tissues was well maintained.

Incorporation of [^{3}H]thymidine into carcinogen-treated explants during the first 9 days of culture occurred throughout the crypts and in the surface epithelium (Fig. 1). Carcinogen-free medium controls incorporated [^{3}H]thymidine into the lower one-third of the crypts (Fig. 2). The number of cells synthesizing DNA during the 4-hr [^{3}H]thymidine pulse increased after 3 days in culture (1 MNNG exposure) and remained between 28 and 40% for the first 9 days (Table II). No label was detected 15 days after initial MNNG exposure.

Nine days after initial MNNG exposure, all treated explants produced outgrowth of epithelioid cells into the lacunae of the fibrin foam (Figs. 3 and 4). Explants exposed to 1.0 μg MNNG/ml showed greater ability to grow into the matrix as compared with explants treated with 0.1 or 10.0 μg MNNG/ml. The degree of penetration into the matrix correlated with increased proliferative activity of the tissue. Evidence of proliferation was provided by identification with autoradiography of cells that have synthesized DNA (Fig. 5).

After 15 and 21 days in culture (5 and 7 MNNG exposures), extensive degeneration and necrosis were noted in explants treated with all concentrations of MNNG, as shown in Table I. DNA synthesis in epithelial crypt cells was also reduced compared to the first 9 days of culture. Outgrowth of epithelioid cells into the fibrin foam matrix was still observed in the 1.0 μg MNNG/ml-treated explants.

Discussion. Organ cultures of colonic mucosa maintained in its differentiated state have usually been limited to short-term studies (8, 9). We have recently described an organ culture model of adult rat distal colonic mucosa

TABLE II. LABELING INDEX OF EPITHELIAL CRYPT CELLS FROM COLONIC CULTURES DISCONTINUOUSLY EXPOSED TO VARIOUS CONCENTRATIONS OF MNNG[a]

Days after initial MNNG exposure	No. of 3-hr exposures	No. of crypts analyzed	Labeling index[b]			
			Control medium	0.1 μg/ml	1.0 μg/ml	10.0 μg/ml
3	1	40	29	31	26	35
9	3	25	28	29	40	30
15	5	25	29	0	0	0
21	7	15	22	0	0	0

[a] Mucosal explants were pulse-labeled with 25 μCi [^{3}H]thymidine/ml (sp act 20 Ci/mmole) for 4 hr immediately before fixation.

[b] The labeling index was determined from longitudinally sectioned crypts by calculating the ratio of labeled cells/total cells × 100. A cell was considered labeled if five or more grains were seen over the nucleus.

that can be maintained for up to 28 days (6). In our system, the colonic mucosa is separated from the muscle layers and cultured on a substrate of human fibrin foam. Maintenance of this differentiated epithelium makes it possible to study the direct effects on colonic mucosa of environmental carcinogens.

The present study shows that MNNG, a powerful mutagenic and carcinogenic substance, produces alterations in epithelial cell function and growth patterns in adult rat distal colon in organ culture. Exposure to 1.0 μg MNNG/ml provided the most significant carcinogenic effects. This is based on the epithelioid outgrowth noted throughout the 21-day observation period, along with the enlargement of the proliferative zone in the colonic crypts. The supplemented Waymouth's MB 752/1 medium with 10% FBS and high oxygen (95%) (6) employed for maintenance of the carcinogen-treated organ cultures also permitted the maintenance of epithelioid cells in outgrowth from organ cultures.

The incorporation of [^{3}H]thymidine throughout the crypts and in the surface epithelium of MNNG-treated cultures is interesting since Lipkin (10) showed that one of the first changes associated with malignancy in the colon is the enlargement of the proliferative zone in the colonic crypts. The reduction in labeling index 15 days after initial MNNG treatment appears to result from cell death and not from inhibition of entry of tritiated thymidine into DNA synthesis. This interpretation is supported by the histological observations in Table I showing extensive degeneration and necrosis in colonic crypts of MNNG-treated explants. These results suggest that in order to examine the cellular effects of carcinogenic compounds in rat colonic mucosa over long periods, it will be necessary to transplant carcinogen-exposed colonic explants into isogenic hosts after 1 or 2 weeks exposure *in vitro*.

Further investigations to characterize the cellular outgrowth from carcinogen-treated explants are needed. By establishing epithelial cell cultures from carcinogen-treated colonic organ cultures, alteration in growth potential can be studied similar to rat tracheal cultures exposed to MNNG (11).

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