[CANCER RESEARCH 36, 1379–1383, April 1976]

Effect of Vitamin A Deficiency on Rat Colon Carcinogenesis by *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine[1]

Tomio Narisawa,[2] Bandaru S. Reddy,[3] Ching-Quo Wong, and John H. Weisburger

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595

SUMMARY

The effect of vitamin A deficiency on the sensitivity of the colon to the carcinogenic effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), a direct-acting carcinogen, given intrarectally was studied in female Fischer rats. Animals maintained on Purina laboratory chow, semipurified vitamin A-free diet, or semipurified vitamine A-supplemented diet were given intrarectally 1.25, 0.63, or 0.31 mg MNNG 3 times weekly for 30 weeks and autopsied at the 45th week. The number of large bowel tumors per tumor-bearing rat was higher in animals receiving 1.25 mg MNNG compared to those given 0.63 or 0.31 mg. Vitamin A deficiency in rats given 1.25 mg MNNG significantly suppressed the large bowel tumor induction compared to rats fed adequate vitamin A. A high incidence of squamous cell papillomatosis of the urinary bladder was observed in rats fed vitamin A-free diet and given 1.25 mg MNNG. The present experiment suggests that the large intestine has a susceptibility that is different from that of the respiratory and urinary tracts to tumorigenic stimulation in vitamin A-deficient status.

INTRODUCTION

Epidemiological studies indicate that colon cancer is associated with environmental factors, and they also suggest that diet, particularly high in fat and meat, may be among the most important factors involved (1, 11, 31–33). Also an elevated consumption of refined carbohydrates has also been incriminated as a causative factor (3).

In addition to these major nutritional elements, micronutrients such as vitamins and minerals are considered to play a modifying role in colon carcinogenesis (29). The study of vitamin A in colon carcinogenesis is of interest because it is known to be involved in the differentiation, maturation, and maintenance of intestinal mucosal epithelium (8). Recently, Newberne and Rogers (21) unexpectedly found that vitamin A-deficient rats developed some colon tumors upon intake of aflatoxin B_1, a liver carcinogen. However, further investigation by Rogers *et al.* (23) demonstrated that neither vitamin A deficiency nor hypervitaminosis A influenced the induction of colon tumors in rats by 1,2-dimethylhydrazine, a carcinogen that required metabolic activation.

This study was designed to investigate the effect of vitamin A deficiency on the sensitivity of the colon to the carcinogenic effect of a direct-acting carcinogen, MNNG[4] administered i.r. to rats. MNNG administered by this method affects the mucosa of the large bowel directly without metabolic activation (19).

MATERIALS AND METHODS

Weanling female CD-Fischer rats (Charles River Breeding Laboratory, Wilmington, Mass.) were randomly divided into 3 groups and fed *ad libitum* one of the following diets: *(a)* Purina laboratory chow; *(b)* semipurified pelleted vitamin A-free diet; or *(c)* the vitamin A-free diet supplemented vitamin A (ICN Nutritional Biochemicals Co., Cleveland, Ohio). The vitamin A-free semipurified diet consisted of the following ingredients: vitamin test casein, 18%, starch, 65%, salt mixture No. 2 U.S.P. XIII, 4%, dried yeast, 8%, viosterol, 10 mg/kg. The vitamin A-supplemented group was fed the vitamin A-free diet and in addition received p.o. 30 IU vitamin A as vitamin A palmitate per g of vitamin A-free diet, based on a weekly measured average consumption 2 or 3 times weekly for the whole experimental period. The body weights were also determined each week. From the 10th week following the start of the experiment a few rats of the vitamin A-free group showing severe vitamin A deficiency were given p.o. a single dose of 10 IU vitamin A in 0.1-ml suspension to prevent death. From the 35th week on, all rats of this group were supplemented with 10 IU vitamin A once a week to prevent death from vitamin A deficiency.

To determine vitamin A-deficient status, the vitamin A level in the serum and liver was measured at the 5th, 10th, 15th, and 45th week. Rats were anesthetized with ether, bled by heart puncture, and sacrificed. Livers were excised immediately and stored at −20° until they were analyzed for vitamin A content. Vitamin A from the livers was extracted with ethyl ether (14) and determined by the trifluoroacetic method (9). Serum vitamin A was measured by the fluorometric method (12).

At the 5th week after the start of the respective diets, rats in each group received i.r. 3 dose levels of MNNG (Aldrich Chemical Co., Milwaukee, Wis.): 0.5 mg of 0.25% MNNG in distilled water (1.25 mg MNNG, high MNNG); 0.5 ml of

[1] Supported in part by USPHS Contract NOI-CP-33208 and Grant CA-15400 from the National Cancer Institute.

[2] Visiting Investigator, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, N. Y. 10595, on leave of absence from Akita University School of Medicine, Akita, Japan.

[3] To whom requests for reprints should be addressed.

Received August 8, 1975; accepted December 19, 1975.

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

0.125% MNNG solution (0.625 mg MNNG, medium MNNG); and 0.5 ml of 0.0625% MNNG solution (0.3125 mg MNNG, low MNNG), 3 times weekly for 30 weeks by the technique described previously (18, 19). Endoscopic examination of colon tumors was routinely done by the methods developed in our laboratory (20).

Moribund rats were killed. All surviving animals were killed at the 45th week. Detailed autopsy was performed on all rats that died or were killed. All organs, including the intestine, were examined grossly and histologically by routine procedures. Both hematoxylin and eosin staining and periodic acid-Schiff staining were used routinely.

RESULTS

Vitamin A-deficient Status. The vitamin A concentrations in the serum and liver in vitamin A-free group were low throughout the experiment (Table 1). After the 7th week, the body weights were lower in rats fed vitamin A-free diet compared to those fed vitamin A-supplemented diet. The vitamin A-free group consumed 5 to 15% less diet compared to the vitamin A-supplemented groups.

Histopathological examination of various organs indicated that keratinizing squamous cell metaplasia of the mucosa of the respiratory and urinary tracts was marked in rats fed vitamin A-free diet (Table 2). Many cases of squamous cell papillomatosis with keratinizing squamous cell metaplasia were observed in the bladder (Table 2). Carcinoma of the bladder was not present. However, high-MNNG rats showed significantly higher incidence of the metaplasia and papillomatosis of the bladder ($p < 0.05$).

Although few animals fed vitamin A-deficient diet showed an inconsistent reduction of goblet cells in the small and large intestine, these changes were not uniformly observed in the intestine of vitamin A-deficient rats in contrast to observations on cells in the respiratory and urinary tracts. Now the question is whether the intestinal epithelial cells were actually in a vitamin A-deficient state in the animals fed deficient diet; however, the clinical data, namely the serum

Table 1

Serum and hepatic concentration of vitamin A in rats with different feeding regimen

Feeding regimen	Time examined (wk)[a]	No. of rats examined	Vitamin A concentration: Serum (μg/100 ml)	Vitamin A concentration: Liver (μg/g, wet wt)
Vitamin A-free	5	2	13.7[b]	3.1[b]
	10	2	8.8	2.0
	15	2	4.6	
	45	6	8.5 ± 1.0[c]	2.5 ± 0.2[c]
Vitamin A-supplemented	5	2	33.2	80.3
	10	1	50.0	99.0
	15	2	66.0	
	45	6	78.6 ± 3.7	100.4 ± 3.8
Purina laboratory chow	5	2	80.7	179.0
	10	2	82.0	114.0
	15	2	89.6	
	45	6	81.9 ± 3.3	113.2 ± 4.9

[a] Weeks after the start of the experiment.
[b] Average value.
[c] Mean ± S.E.

Table 2

Incidence of squamous cell metaplasia and urinary bladder papillomatosis in rats of vitamin A-free group dead between 36th and 45th week

MNNG dose[a]	No. of effective rats	No. of rats with metaplasia in: Trachea (%)	No. of rats with metaplasia in: Urinary bladder (%)	No. of rats with urinary bladder[b] papillomatosis (%)
Vitamin A-free				
High-MNNG	24	13 (54)	18 (75)[c]	16 (67)[d]
Medium-MNNG	24	10 (42)	12 (50)	8 (33)
Low-MNNG	13	4 (31)	6 (46)	3 (23)
No-MNNG	7	5 (71)	2 (29)	1 (14)

[a] High-MNNG, 1.25 mg MNNG; medium-MNNG, 0.625 mg MNNG; low-MNNG, 0.3125 mg MNNG. These MNNG doses in 0.5-ml solution were given i.r. 3 times weekly for 30 weeks. No-MNNG, no i.r. treatment.
[b] All urinary bladder papillomatosis appeared in urinary bladders with squamous cell metaplasia.
[c] Significantly higher than non-MNNG group ($p < 0.05$).
[d] Significantly higher than other 3 groups ($p < 0.05$).

and liver vitamin A levels, indicate that these animals were under conditions of vitamin A deficiency. On the basis of intestinal histological as well as clinical evaluation, it is possible that these animals were on marginal vitamin A-deficiency state. Neither squamous cell metaplasia of respiratory and urinary tracts nor papillomas or papillomatosis of the urinary bladder were detected in rats fed Purina laboratory chow or vitamin A-supplemented semipurified diet.

Large Bowel Neoplasms. At the 40th week (5 weeks after the end of MNNG administration) large bowel tumors were found by endoscopy in 5 of 11 rats examined in the vitamin A-free high-MNNG group and in 22 of 23 in the Purina laboratory chow high-MNNG group, respectively. On the basis of this information, all surviving rats were killed by the 45th week and the experiment was terminated.

Table 3 lists the incidence and number of large bowel neoplasms of rats in each group that died or were sacrificed during the 40th to 45th week. In the Purina laboratory chow group, the number of tumors per large bowel tumor-bearing rat was significantly higher in high-MNNG than in medium-MNNG and low-MNNG rats ($p < 0.05$). In the vitamin A-free and vitamin A-supplemented groups, the tumor incidences of high-MNNG rats were also significantly higher than those of medium- and low-MNNG groups.

The tumor incidence of the vitamin A-free high-MNNG group was one-half that of the vitamin A-supplemented high-MNNG group. The tumor incidences of the vitamin A-free medium-MNNG and vitamin A-free low-MNNG groups were low and similar to those of the vitamin A-supplemented medium-MNNG and vitamin A-supplemented low-MNNG groups, respectively. The cumulative tumor incidence of all rats with different MNNG doses in the vitamin A-free group (26%) was significantly less ($p < 0.0$) than that of vitamin A-supplemented group (50%). The number of tumors per tumor-bearing rat was lower in the vitamin A-free high-MNNG group than in the vitamin A-supplemented high-MNNG group.

All large bowel neoplasms were confined to the distal half of the large bowel exposed to the instilled solutions. All neoplasms showed polyp or polypoid shape in various sizes. The diameter of adenocarcinomas ranged from 0.2 to 0.4 cm in the vitamin A-free group, from 0.1 to 0.7 cm in the vitamin A-supplemented group, and from 0.2 to 1.6 cm in the Purina laboratory chow group; and the diameter of the adenomas ranged up to 0.8, 0.7, and 1.2 cm in rats on these 3 diets, respectively. The tumors of rats with semisynthetic diet and/or vitamin A deficiency appeared to be somewhat smaller in diameter. Histopathological evaluation showed that, of all 77 colorectal carcinomas, 76 were well-differentiated adenocarcinomas, and one was a signet-ring cell carcinoma. All carcinomas invaded the submucosa and 10 involved extensively in the tunica muscularis propria or serosa. Metastasis to the mesenteric lymph nodes was found in only 1 adenocarcinoma. There were no distinguishable differences in the microscopic appearance of the neoplasm among groups with different diets.

DISCUSSION

Vitamin A inhibited the induction of tumors in the lung, forestomach, and cervix of hamsters; in the lungs of rats by

Table 3

Large bowel tumor incidence from 40th to 45th week by intrarectal instillation of MNNG in CD-Fischer rats fed deficient and adequate amounts of vitamin A

Feeding regimen and MNNG dose[a]	No. of effective rats	No. of rats with neoplasms (%)	No. of neoplasms		
			Adenocarcinoma	Adenoma	Others
Vitamin A-free					
High-MNNG	20	9 (45)[b]	5	12	2[c,d]
Medium-MNNG	24	4 (17)	1	2	2[d,e]
Low-MNNG	13	2 (15)	0	2	
Vitamin A-supplemented[f]					
High-MNNG	24	23(96)[b]	17	49	4[d,g-i]
Medium-MNNG	22	7(32)	1	10	
Low-MNNG	22	4(18)	0	3	1[g]
Purina laboratory chow					
High-MNNG	23	22 (96)	44	46	2[d,h]
Medium-MNNG	23	17 (74)	9	19	1[j]
Low-MNNG	21	3 (14)	0	4	

[a] High MNNG, 1.25 mg MNNG; medium MNNG, 0.625 mg MNNG; low MNNG, 0.3125 mg MNNG. These MNNG doses in 0.5-ml solution were given 3 times weekly for 30 weeks.
[b] Significantly different between both ($p < 0.001$).
[c] Hemangioma.
[d] Lymphoma.
[e] Fibroma.
[f] Thirty IU vitamin A per g, vitamin A-free diet were given p.o.
[g] Squamous cell carcinoma of anal region.
[h] Squamous cell papilloma of anal region.
[i] Leiomyosarcoma.
[j] Hemangiosarcoma.

carcinogenic polycyclic hydrocarbons (4, 5, 24); and in the skin of mice and rabbits (2, 7, 22, 25). Also, it suppressed the hyperplastic or metaplastic changes of cultured mouse prostate and hamster tracheal epithelium in a medium containing 3-methylcholanthrene (6, 15). Other reports indicate that vitamin A enhanced 3-methylcholanthrene-induced tumors in the lung of hamsters and mice, and the cancer induction of the cheek pouch of hamsters by dimethylbenzanthracene and benzo(*a*)pyrene (16, 26, 27).

Newberne and Rogers (21) demonstrated that aflatoxin B_1 induced colon tumors in vitamin A-deficient Sprague-Dawley rats, while aflatoxin B_1 had no effect on the colon of rats with adequate vitamin A. However, vitamin A deficiency in the same strain of rats failed to affect the colon tumor induction by i.g. administration of 1,2-dimethylhydrazine (22). In our experiment vitamin A deficiency in rats given high MNNG doses significantly suppressed the tumor induction of the large bowel, compared to rats with adequate dietary vitamin A. The vitamin A deficiency was documented by its low concentration in the serum and liver, less body weight gain of rats, and squamous cell metaplasia of the respiratory and urinary tracts. However, in the large and small intestine there was no discernible histological alteration as a decrease of goblet cells (30). The intestinal epithelial cells showed maturation and well-differentiated normal figures in the deficient rats. Thus, it is quite probable that the large bowel mucosa was capable of absorbing i.r. administered MNNG.

Another important point that should be considered is that the animals fed vitamin A-deficient diet consumed less food and weighed less than control rats fed vitamin A-supplemented diet. The experimental evidence suggests that caloric restriction inhibits tumor incidence in experimental animals (28). Since the animals fed vitamin A-deficient diet consumed less calories than did control animals fed vitamin A-supplemented diet, the observed effects in the vitamin A-deficient group could be due to vitamin A deficiency and/or caloric restriction.

We noted a high incidence of squamous cell papillomatosis of the urinary bladder in the vitamin A-free high-MNNG group. Also, there were more rats with urinary bladder metaplasia in the high-MNNG group compared to the no-MNNG group. About 90% of MNNG and metabolites is excreted in the urine within 24 hr (14). Thus, the metaplastic change of the urinary bladder stemming from the vitamin deficiency was likely to be followed by a tumorigenic stimulation of MNNG absorbed from the large bowel, which may have been the cause of the increased urinary bladder papillomatosis. The metaplastic change might predispose the epithelium to neoplastic changes by exposure to carcinogenic stimulation (5, 24). Along these lines, there was a higher binding of isotope from benzo(*a*)pyrene with DNA of basal cells located in squamous metaplastic areas than in normal areas of trachea of vitamin A-deficient animals (10). Our experiment suggests that the intestine has different susceptibility than the respiratory and urinary tracts to tumorigenic stimulations in vitamin A-deficient environment. The influence of vitamin A on experimental carcinogenesis was reviewed recently (17). It would appear that little is known about the role of vitamin A in normal differentiation. The cancer-potentiating effects of a vitamin A deficiency need further study.

REFERENCES

1. Berg, J. W., Howell, M. A., and Silverman, S. J. Dietary Hypotheses and Diet-related Research in Etiology of Colon Cancer. Health Serv. Rept., *88:* 915–924, 1973.
2. Bollag, W. Prophylaxis of Chemically Induced Benign and Malignant Epithelial Tumors by Vitamin A Acid (Retinoic Acid). European J. Cancer, *8:* 686–693, 1972.
3. Burkitt, D. P. Large-Bowl Cancer: An Epidemiologic Jigsaw Puzzle. J. Natl. Cancer Inst., *54:* 3–6, 1975.
4. Chu, E. W., and Malmgren, R. A. An Inhibitory Effect of Vitamin A on the Induction of Tumors of Forestomach and Cervix in the Syrian Hamster by Polycyclic Hydrocarbons. Cancer Res., *25:* 884–895, 1965.
5. Cone, M. V., and Nettesheim, P. Effects of Vitamin A on 3-Methylcholanthrene-Induced Squamous Metaplasia and Early Tumors in the Respiratory Tract of Rats. J. Natl. Cancer Inst., *50:* 1599–1606, 1973.
6. Crocker, T. T., and Sanders, L. L. Influence of Vitamin A and 3,7-Dimethyl-2,6-octadiental (Citral) on the Effect of Benzo(*a*)pyrene on Hamster Trachea in Organ Culture. Cancer Res., *30:* 1312–1318, 1974.
7. Davies, R. E. Effect of Vitamin A on 7,12-Dimethylbenz(a)anthracene-induced Papillomas in Rhino Mouse Skin. Cancer Res., *27:* 237–241, 1967.
8. DeLuca, L., Schumacher, M., and Wolf, G. Biosynthesis of a Fucose Containing Glycopeptide from Rat Small Intestine in Normal and Vitamin A Deficient Conditions. J. Biol. Chem., *245:* 4551–4566, 1970.
9. Dugan, R. E., Fregerio, N. A., and Siebert, J. M. Calorimetric Determination of Vitamin A and Its Derivatives with Trifluoracetic Acid. Anal. Chem., *35:* 114–117, 1964.
10. Genta, V. M., Kaufman, D. G., Harris, C. C., Smith, J. M., Sporn, M. D., and Saffiotti, U. Vitamin A Deficiency Enhances Binding of Benzo(*a*)pyrene to Tracheal Epithelial DNA. Nature *247:* 48–49, 1974.
11. Haenszel, W., Berg, J. W., Segi, M., Kurihara, M., and Lucke, F. B. Large-Bowel Cancer in Hawaiian Japanese. J. Natl. Cancer Inst., *51:* 1765–1774, 1973.
12. Hansen, L. G., and Warwick, W. J. A Fluorometric Method for Serum Vitamin A. Am. J. Clin. Pathol., *50:* 525–529, 1968.
13. Hinds, F. C., Peter, A. P., and Richards, G. E. Methods of Extracting Vitamin A from Liver. J. Animal Sci., *27:* 1678–1681, 1968.
14. Kawachi, T., Kogure, K., Kamijo, Y., and Sugimura, T. The Metabolism of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Rats. Biochim. Biophys. Acta, *222:* 409–415, 1970.
15. Lasnitzki, I., and Goodman, D. S. Inhibition of the Effects of Methylcholanthrene on Mouse Prostate in Organ Culture by Vitamin A and Its Analogs. Cancer Res., *34:* 1564–1571, 1974.
16. Levij, I. S., and Palliack, A. Potentiating Effect of Vitamin A on 9,10-Dimethyl-1,2-benzanthracene-carcinogenesis in the Hamster Cheek Pouch. Cancer, *22:* 300–306, 1968.
17. Maugh, T. H. Vitamin A: Potential Protection from Carcinogenesis. Science, *186:* 1198, 1975.
18. Narisawa, T., Magadia, N. E., Weisburger, J. H., and Wynder, E. L. Promoting Effect of Bile Acids on Colon Carcinogenesis after Intrarectal Instillation of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Rats. J. Natl. Cancer Inst., *53:* 1093–1097, 1974.
19. Narisawa, T., Nakano, H., Hayakawa, M., Sato, T., and Sakuma, A. Tumors of the Colon and Rectum Induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *In:* W. Nakahar, S. Takayama, T. Sugimura, and S. Odashima (eds.), Topics in Chemical Carcinogenesis, pp. 145–158. Baltimore: University Park Press, 1972.
20. Narisawa, T., Wong, C. Q., and Weisburger, J. H. Evaluation of Endoscopic Examination of Colon Tumors in Rats. Am. J. Digest Diseases, *20:* 928–934, 1975.
21. Newberne, P. M., and Rogers, A. E. Rat Colon Carcinomas Associated with Aflatoxin and Marginal Vitamin A. J. Natl. Cancer Inst., *50:* 439–448, 1973.
22. Prutkin, L. Antitumor Activity of Vitamin A Acid and Fluoracil Used in Combination on the Skin Tumor, Keratoacanthoma. Cancer Res., *33:* 128–133, 1973.
23. Rogers, A. E., Herdon, B. J., and Newberne, P. M. Induction by Dimethylhydrazine of Intestinal Carcinoma in Normal Rats and Rats Fed High or Low Levels of Vitamin A. Cancer Res., *33:* 1003–1009, 1973.
24. Saffioti, U., Montesano, R., Sellakumar, A. R., and Borg, S. A. Experimental Cancer of the Lung. Inhibition by Vitamin A of the Induction of Tracheobronchial Squamous Metaplasia and Squamous Cell Tumors. Cancer, *20:* 857–864, 1967.
25. Shamberger, R. J. Inhibitory Effect of Vitamin A on Carcinogenesis. J. Natl. Cancer Inst., *47:* 667–673, 1971.
26. Smith, D. M., Rogers, A. E., Herndon, B. J., and Newberne, P. M. Vitamin A (Retinyl Acetate) and Benzo(*a*)pyrene-induced Respiratory Tract Carcin-

ogenesis in Hamsters Fed a Commercial Diet. Cancer Res., *35:* 11–16, 1975.
27. Smith, W. E., Yazdi, E., and Miller, L. Carcinogenesis in Pulmonary Epithelia in Mice on Different Levels of Vitamin A. Environ. Res., *5:* 152–163, 1972.
28. Tannenbaum, A. Nutrition and Cancer. *In:* F. Homburger (ed.), The Physiopathology of Cancer, Ed. 2, pp. 517–562. New York: Hoeber-Harper, 1959.
29. Weisburger, J. H. Chemical Carcinogenesis in the Gastrointestinal Tract. *In:* American Cancer Society (ed.), Seventh National Cancer Conference Proceedings, pp. 465–473. Philadelphia: J. B. Lippincott, 1973.
30. Wolbach, S. B., and Howe, P. R. Tissue Changes following Deprivation of Fat Soluble A Vitamin. J. Exptl. Med., *42:* 753–777, 1925.
31. Wynder, E. L., Kajitani, T., Ishikawa, S., Dodo, H., and Takano, A. Environmental Factors of Cancer of the Colon and Rectum. II. Japanese Epidemiological Data. Cancer, *23:* 1210–1220, 1969.
32. Wynder, E. L., and Reddy, B. S. Metabolic Epidemiology of Colorectal Cancer. Cancer, *34:* 801–806, 1974.
33. Wynder, E. L., and Shigematsu, T. Environmental Factors of Cancer of the Colon and Rectum. Cancer, *20:* 1520–1561, 1967.

Effect of Vitamin A Deficiency on Rat Colon Carcinogenesis by *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine

Tomio Narisawa, Bandaru S. Reddy, Ching-Quo Wong, et al.

Cancer Res 1976;36:1379-1383.