Protective Effect of β-Carotene Against Colon Tumors in Mice [1,2]

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ABSTRACT—The effect of dietary β-carotene on colon carcinogenesis induced by 1,2-dimethylhydrazine [(DMH) CAS: 540-73-8] was studied in female inbred Swiss Webster (ICR) mice. At age 10 weeks and continuing throughout the experiment, mice received diets consisting mainly of natural foods (laboratory chow) and containing 2 or 22 mg β-carotene/kg. At age 15 weeks they received 7 weekly sc injections of DMH (total dose: 196 mg DMH·diHCl/kg body wt). When autopsied 31 weeks after the first DMH injection, the incidence (percent of mice with tumors) and multiplicity (number of tumors/tumor-bearing mouse) of colon tumors were reduced by half in the mice supplemented with β-carotene. There was a much greater decrease in adenocarcinomas than in adenomas. Mice observed for 13 additional weeks revealed that the mortality rate, due largely or wholly to colon cancer, was only about half in supplemented mice. Mice sacrificed 12 weeks after the first dose of DMH (i.e., well before tumors appeared) showed mild colon mucosal hyperplasia. β-Carotene supplementation, however, did not alter this, indicating that the protective effect against colon cancer may have occurred at a late stage of carcinogenesis.—JNCI 1987; 78:1211-1214.

In 1981, Peto et al. (*1*) reinterpreted various evidence, particularly that from prospective and case-control studies, and theorized that dietary β-carotene has a preventive role against cancer. The evidence on which they based this concept pertains largely to lung and gastrointestinal cancer. Unfortunately, these studies generally measured, not β-carotene per se, but an index of it, namely total vitamin A or "vegetables." Three subsequent studies of human lung cancer, in each of which β-carotene was specifically measured, have provided supporting evidence (*2-4*). However, one of these reports, a prospective study from Chicago (*4*), and a recent Australian case-control study (*5*) failed to confirm this for cancer of the colon and rectum. Other evidence that β-carotene prevents cancer has come from experimental animal studies. Thus the carotenoid has been found to have protective effects in rats and mice against tumors at various sites (*6*) including the skin (*7*) and the salivary gland (*8*). It was also protective in hamsters against carcinoma of the buccal pouch (*9*) and in mice inoculated with adenocarcinoma cells (*10*). Most of these studies used very high levels of β-carotene.

The present study investigated the effect of dietary β-carotene at a nutritionally relevant level (20 mg/kg diet) against colon tumors in mice induced by DMH. The parameters monitored included tumor incidence, multiplicity, survival time, and colon mucosal hyperplasia.

MATERIALS AND METHODS

Animals and diets.—Female Swiss Webster inbred (ICR) mice were purchased from Charles River, St. Constant, Quebec. At age 10 weeks, they were placed on the experimental diets (mean weight, 32 g; range, 26-41 g). The diets (as dry weight) contained 1.9% corn oil (Mazola; Best Foods, Montreal), 98.1% Wayne Rodent Blox meal (Continental Grain Co., Chicago, IL), and 2 or 22 mg β-carotene/kg (type III; Sigma Chemical Co., St. Louis, MO). β-Carotene was first dissolved in the corn oil. The diets were mixed with an equal weight of water. Food and water were provided ad libitum. Food was given every 1-2 days (usually daily) from a stock prepared roughly every 5 days and stored at 5°C. Analysis of food indicated that under these conditions there was little loss of β-carotene. Wayne Rodent Blox meal consists mainly of cereal and vegetable foods and contains 24% protein, 3.6% crude fiber, 4.1% fat, 15 IU vitamin A/g, and an adequate concentration of all nutrients.

Treatment.—Where indicated, mice were given sc injections of DMH commencing after 5 weeks of dietary treatment. DMH (Sigma Chemical Co.) was dissolved in 1 m*M* EDTA and neutralized with saturated sodium bicarbonate. The total dose (196 mg DMH·diHCl/kg body wt) was given in 7 weekly injections (20.5, 24.5, 27, 31, 31, 31, and 31 mg/kg). Giving a gradually rising dose is based on the observation that mice develop tolerance to DMH and thus allows the number of injections to be reduced while minimizing toxic effects (*10*).

Mice were divided into the following groups: *a*) Group 1 (pretumor mice). Four subgroups (2 or 22 mg β-carotene/kg; each with or without DMH treatment). These were sacrificed 12 weeks after the first DMH injection. *b*) Group 2 (tumor mice). Two subgroups (2 or 22 mg β-carotene/kg; all mice DMH treated) were sacrificed 31 weeks after the first DMH injection. *c*) Group 3

ABBREVIATIONS USED: DMH = 1,2-dimethylhydrazine; H & E = hematoxylin and eosin.

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TABLE 1.—*Colon mucosal DNA content and crypt size in relation to DMH and β-carotene*[a]

DMH	Dietary β-carotene, mg/kg	No. of mice	Body wt, g[b]	DNA-protein, μg/mg[b]	DNA-wet wt, μg/mg[b]	No. of cells/ crypt column[b]	Crypt column length, μm[b]
–	2	10	37.0±3.7	50.4±7.1[c]	3.44±.62	21.6±1.4[c]	187±21
–	22	11	35.9±2.9	53.1±3.9	3.47±.49	20.9±3.5[c]	171±25[d]
+	2	10	36.2±2.1	58.2±9.6[c]	3.78±.35	24.8±4.6[c]	200±25
+	22	12	36.3±3.0	52.6±7.6	3.74±.53	25.6±4.5[c]	229±51[d]

[a] Mice are of group 1 (sacrificed 12 wk after first dose of DMH).
[b] Values are means ± SD. Statistical comparisons were made only between values differing in only one factor (DMH or β-carotene). Values sharing the same suffix are significantly different by Student's *t*-test.
[c] $P<.05$.
[d] $P<.01$.

(survival mice). This group underwent the same procedure as group 2 but was left for 13 additional weeks. Mice showing signs of advanced cancer (severe weight loss or moribund, usually associated with visible tumor growth or anal bleeding) were sacrificed and examined.

Hyperplasia.—This was quantitated in mice of group 1 only. Histologic assessment: A portion of colon 1-2 cm from the anus was removed, flushed with saline, and placed in neutral buffered Formalin. It was sectioned longitudinally and stained (H & E). On each slide, the height of five separate crypt columns was measured as was the number of cells lining one side of the crypt column (*11*).

Biochemical analysis: The remaining colon (excluding the cecum) was opened longitudinally. The distal half was washed under the tap, and the mucosal layer was scraped off with the use of microscope slides. This separation technique was confirmed histologically. The scrapings were weighed and homogenized in 4 ml 0.06 *M* potassium phosphate (pH 7.8). The homogenate was analyzed for DNA by the method of Burton (*12*) as modified by Giles and Myers (*13*) and for protein (*14*).

Tumor assessment.—Mice were sacrificed and internal organs grossly examined. The colon (including the cecum) was opened and carefully examined. Suspect tumors were removed and placed in neutral buffered Formalin. They were confirmed after staining (H & E) (*15, 16*). The above procedures were performed by an observer who was unaware as to which dietary group samples and mice had come from.

Other analyses.—Malonaldehyde was measured in samples of liver and colon mucosa by the thiobarbituric acid method of Beuge and Aust (*17*). Superoxide dismutase in hepatic postmitochondrial supernatant (essentially Cu, Zn activity) was assayed by measuring the reduction of nitro blue tetrazolium after generating superoxide ions with xanthine and xanthine oxidase as described by Oberley et al. (*18*).

Statistical analyses.—Data were analyzed by two-tailed Student's *t*-test and by chi square. Mouse survival was analyzed by the log rank method of Peto et al. (*19*). *P*-values less than .05 were considered significant.

RESULTS

Body weight.—In the 12 weeks following the first DMH injection, weight was apparently unaffected by dietary level of β-carotene or by DMH treatment (table 1). However, during the following 19 weeks, β-carotene-supplemented mice became significantly heavier than unsupplemented mice (table 2). No relationship was seen between weight and the presence of colon tumors except that mice with terminal cancer often experienced severe weight loss (data not shown).

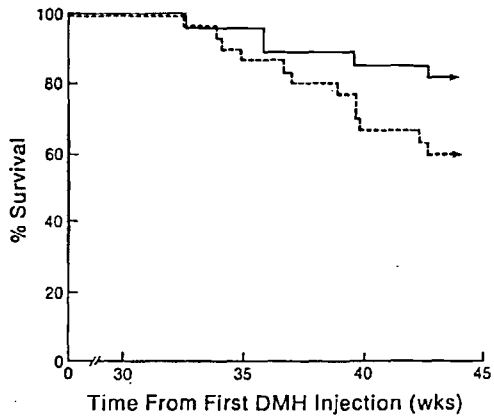
Food intake.—This was measured 10-11 weeks after the first injection. The average food intake (dry weight) was 4.2 g/mouse/day and was not affected by diet or DMH treatment.

Hyperplasia.—In mice sacrificed 12 weeks after the first DMH injection (group 1), DMH treatment induced mild hyperplasia of the colon mucosa (table 1). This was much more apparent with the use of histologic

TABLE 2.—*Effect of dietary β-carotene on the incidence and type of colon tumors in mice treated with DMH*[a]

Dietary β-carotene, mg/kg	No. of mice	Weight, g[b]	Percent of mice with colon tumors, tumor incidence			Colon tumors/tumor-bearing mouse, tumor multiplicity[b]		
			Total	Adenoma	Adenocarcinoma	Total	Adenoma	Adenocarcinoma
2	31	34.4±3.4[c]	74.2[d]	61.2	32.3[c]	2.52±2.00[f]	1.96±1.72[c]	0.57±0.73[c]
22	32	37.0±4.0[c]	37.5[d]	37.5	3.1[c]	1.25±0.45[f]	1.17±0.39[c]	0.08±0.29[c]

[a] Mice are of group 2 (sacrificed 31 wk after first dose of DMH).
[b] Values are means ± SD.
[c] Significantly different by Student's *t*-test, $P<.05$.
[d] Significantly different by chi-square, $P<.05$.
[e] Significantly different by chi-square, $P<.01$.
[f] Significantly different by Student's *t*-test, $P<.01$.



TEXT-FIGURE 1.—Life table of survival of group 3 mice as a function of time. Mice were fed diets low (– – –) or high (———) in β-carotene (n = 30 and 27, respectively). Survival was not significantly different between the 2 groups (P = .08).

measurement (number of cells per crypt column and crypt column length) than when measured biochemically (DNA content). β-Carotene did not appear to moderate this hyperplasia.

Tumor data.—The tumor data on mice sacrificed 31 weeks after the first DMH injection (group 2) are shown in table 2. Mice supplemented with β-carotene had far fewer colon tumors. Adenomas were reduced by about 40% in terms of both incidence (percent of mice with a tumor) and multiplicity (tumors per tumor-bearing mouse). Adenocarcinomas were even more dramatically reduced: 96% for incidence and 86% for multiplicity. The great majority of tumors were found between 0.5 and 4.5 cm from the anus (i.e., in the distal colon). The remainder were in the midcolon.

Text-figure 1 depicts the survival of group 3 mice. They were treated as group 2 mice except that they were observed for 13 additional weeks but were sacrificed if they showed signs of advanced cancer. As far as could be determined, colon cancer caused most, if not all, deaths. β-Carotene-treated mice had about half the mortality rate of unsupplemented mice (18.5% vs. 40.0%). However, this did not achieve statistical significance (P = .08).

DISCUSSION

The experiment described here demonstrates that β-carotene has a strong inhibitory action against DMH-induced colon tumors. Both the incidence and multiplicity of tumors were reduced by about half. While adenomas, the predominant type of tumor, were about 40% less common in supplemented mice, adenocarcinomas were largely absent. These observations are based on mice sacrificed 31 weeks after the first DMH injection. We also studied mice treated as above but that were observed for 13 additional weeks. Consistent with the above data, β-carotene-supplemented mice had only about half the mortality of unsupplemented mice, although not significant.

Since the level of supplementary β-carotene (20 mg/kg diet) was within the nutritionally relevant range (equivalent to 150–300 g carrots/3,000 kcal), the results are of importance to the human situation. The results therefore support the concept of Peto et al. (*1*) that β-carotene is protective against cancer.

Compared with human studies, experimental animal investigations have yielded more consistent information as to the protective effect of β-carotene. Thus dietary supplements of this nutrient have blocked tumor formation at several sites in rats, mice, and hamsters (*6–9, 20*). Nonetheless, no reports have yet appeared involving the intestinal tract. The supplementary dose of β-carotene (20 mg/kg diet) is the lowest yet shown to be effective.

Since β-carotene was fed throughout the experiment, it is not known whether the beneficial effect occurred at the initiation or promotion stages. Suda et al. (*9*) studied carcinogenesis in hamster buccal pouch and observed that topical β-carotene had a strong preventive action at both stages of carcinogenesis.

DMH treatment of mice is known to induce colon mucosal hyperplasia (*21–23*), and this was also seen here. Thus when measured 12 weeks after the first of seven weekly DMH injections, the colon mucosa had crypts that were longer and had more cells. To a lesser extent the colon mucosa also had a higher DNA content (per unit wet weight or protein). β-Carotene was ineffective in moderating this hyperplasia. Since hyperplasia is associated with tumor formation, this suggests that β-carotene had not yet exerted its protective effect. The critical period for β-carotene feeding may therefore be late promotion.

A possible mechanism by which β-carotene is active is by stimulating the immune system as suggested by Suda et al. (*9*). Brevard et al. (*24*) observed that β-carotene enhances the immune response of rat colorectal tissue. Similarly, Schwartz et al. (*25*) reported that β-carotene treatment significantly increased the cytotoxicity of macrophages toward hamster tumor cells. An immune-mediated protective effect against colon cancer is consistent with the observation that β-carotene is apparently effective in late promotion.

It is unlikely that the protective effect of β-carotene reflects its vitamin A activity to any great extent. First, since the diets were adequate in preformed vitamin A, it is doubtful that enough β-carotene was converted to vitamin A to appreciably raise the total intake (*26*). Second, although vitamin A has shown some protective ability against colon cancer in rats (*27, 28*), this was much weaker than that seen here with the use of β-carotene.

Evidence indicates that β-carotene may have an antioxidant action, particularly at the relatively low oxygen partial pressures found in most tissues under physiologic conditions (*29*). In particular, it purportedly protects against oxygen radicals. Liver samples were therefore analyzed for malonaldehyde, an index of lipid peroxidation, and for superoxide dismutase. Neither parameter was apparently affected by dietary β-carotene or by the presence of colon tumors.

In other experiments we observed that injection of

DMH 14 hours before sacrifice or feeding β-carotene (500 mg/kg diet) did not affect liver or colon mucosal levels of malonaldehyde (unpublished observations). This suggests that in the conditions of our experiment β-carotene did not act as an antioxidant. However, malonaldehyde lacks specificity as an index of lipid peroxidation (*18, 30, 31*) and β-carotene acts as an antioxidant mainly at low oxygen pressures (*29*); this factor was not controlled during our malonaldehyde assays. It is quite possible, therefore, that parameters of tissue free radical chemistry that we did not measure may have been affected by β-carotene.

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