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Alterations in Colonic Thymidine Kinase Enzyme Activity Induced by Consumption of Various Dietary Fibers (42778)

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Abstract. Dietary fibers may tend to enhance or inhibit chemically induced experimental colon cancer, depending on the particular fiber consumed. This study examined the relationship between colonic thymidine kinase enzyme activity and mucin histochemistry and the reported effects of various dietary fibers on chemically induced colon carcinogenesis. Fiber-supplemented diets containing fibers reported to inhibit (wheat bran) or enhance (guar gum, carrageenan) chemically induced colon carcinogenesis in the rat were selected. Four groups of male Fischer 344 rats consumed 10% wheat bran, 5% guar gum, 5% carrageenan, or fiber-free diets *ad libitum* for 4 weeks. At the completion of the treatment period, the distal 12 cm of colonic mucosa was scraped off and homogenized for determination of thymidine kinase activity, and a 0.5-cm section of midcolon was processed by the high-iron diamine/Alcian blue method for mucin histochemistry. Final animal weights did not differ significantly among groups. Thymidine kinase enzyme specific activity (μmole thymidine phosphate formed $\times 10^6$/min/mg protein, means $\pm$ SEMs) was not significantly different in the fiber-free, wheat bran, and guar gum groups (10.98 ± 1.50, 7.41 ± 1.09, and 9.11 ± 2.04, respectively) but was markedly elevated at 41.84 ± 4.65 in the carrageenan group ($\alpha < 0.001$). Mucin histochemistry failed to reveal any significant differences among dietary groups.

Dietary fibers are a very heterogeneous group of substances, with widely differing chemical and physiological properties. Therefore it is not surprising that these fibers have different effects on the incidence and time of appearance of rat colonic tumors induced by carcinogens such as 1,2-dimethylhydrazine (DMH). In this animal model, the effect of a dietary fiber on the promotion stage of colonic tumor development is generally examined, with DMH or other chemical carcinogens acting as the initiator. Some fibers, such as wheat bran (1), appear to be antipromotors, whereas others, such as carrageenan (2), have been reported to enhance the yield of chemically induced tumors.

Development of short-term methods to predict the likely impact of a dietary fiber on tumor promotion would be useful in planning long-term studies of fiber and cancer promotion. To this end, the present study was undertaken. Thymidine kinase enzyme activity was measured in the colonic mucosa as a marker of cell proliferation (3). Enhanced levels of thymidine kinase have been reported in various rapidly proliferating tissues, including regenerating liver (4) and leukemic cells (5), as well as neoplastic colonic mucosal cells (3). Increased cell proliferation has been documented after feeding fiber-containing diets which promote tumor development (6) or after treatment with various bile acids known to promote the development of colonic tumors (7). The thymidine kinase enzymatic method was chosen over autoradiography because of greater rapidity and ease of quantitation. Previous studies (8) have also shown that various dietary fibers have differential effects upon small intestine thymidine kinase activity. Mucin histochemistry was also examined as determined by the high-iron diamine/Alcian blue staining technique of Spicer (9). A shift in the proportion of mucin toward the unsulfated mucins is considered by some to be an early event in tumorigenesis preceding histologic evidence of malignancy (10). Dietary fiber consumption has also been shown to alter colonic mucin histochemistry (11).

To evaluate these two potential correlates of the tumor-promoting characteristics of dietary fibers, three different fiber sources with previously reported effects on tumor promotion were selected for a 4-week feeding study. Wheat bran was chosen as representative of fibers generally reported to inhibit experi-

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mental colon tumorigenesis; guar gum and undegraded (high-molecular-weight) carrageenan were chosen as fiber sources reported to enhance tumorigenesis. Colonic mucosal thymidine kinase specific activity and mucin histochemistry were measured in rats at the conclusion of the 4-week feeding period.

Materials and Methods. *Animals.* Thirty-two virus antibody-free male Fischer 344 rats (8–9 weeks old, weighing 141–196 g) were obtained from Harlan–Sprague–Dawley, Indianapolis, Indiana. The rats were quarantined for 1 week after receipt, during which they consumed Purina No. 5002 rodent chow and tap water *ad libitum.* Rats were then distributed by weight into four groups to produce groups of eight rats each with similar mean weights and weight ranges. Animals were housed individually in suspended stainless steel wire-bottomed cages over pans filled with hardwood chips. The animal room was equipped with a 12 hr light–dark cycle timer. Temperature and humidity limits were specified at 18–26°C and 30 to 70%, respectively. Overall animal care conformed with Public Health Service and institutional guidelines.

Experimental diets. Four experimental diets were prepared in pelleted form according to our specifications by Dyets, Inc, Bethlehem, Pennsylvania. The composition of the fiber-free control diet, employed in previous studies (12), is shown in Table I. The three dietary fiber sources were (i) wheat bran (American Association of Cereal Chemist's certified hard red wheat bran from the American Association of Cereal Chemists, St. Paul, MN), (ii) undegraded carrageenan (Viscarin GP 109, food grade material consisting mainly of λ-carrageenan from *Gigartina* species, approximate molecular weight 300,000, from FMC Corp., Springfield, NJ), and (iii) guar gum (from ICN Nutritional Biochemicals, Cleveland, OH). These were incorporated into the diets by combining them with the control diet at either a 10% (wheat bran) or a 5% level (guar gum, carrageenan) by weight. These levels were chosen to provide similar levels of dietary fiber in each of the fiber-containing diets, as well as to promote comparable weight gain among the different dietary groups. Avoidance of poor weight gain is particularly important when examining cell proliferation, as starvation has been shown to greatly diminish colonic cell proliferation (16).

TABLE I. COMPOSITION OF FIBER-FREE RAT DIET

Ingredient	%
Casein, edible	19.8
DL-Methionine	0.2
Corn oil	5.0
Bernhart–Tomarelli salt mixture (13), modified	4.0
Vitamin mixture[a]	1.0
Dextrose	34.9
Sucrose	34.9
Choline bitartrate	0.2

[a] Modified from AIN76A (14, 15) to contain increased levels of menadione, biotin, and folic acid, with added inositol to compensate for diminished colonic bacterial synthesis of these compounds occurring in animals fed this diet.

Fiber sources and diets were analyzed for total dietary fiber content (17) before feeding. All analyses, except for the wheat bran (a total dietary fiber analysis was provided with the material by the American Association of Cereal Chemists), were performed by Nutrition International, East Brunswick, New Jersey. Total Dietary Fiber (TDF) contents (as % of the total weight) for the fiber sources were 42.7% for wheat bran, 84.2% for guar gum, and 71.3% for carrageenan. Analysis of the pelleted diets for total dietary fiber content before and after feeding revealed that the desired percentages of fiber had been incorporated into the diets, and that the dietary fibers studied were stable when mixed into the diets for the duration of the study.

Experimental diet feeding period. All animals consumed their respective experimental diets and tap water *ad libitum* for 4 weeks. Animal weights and food consumption were measured weekly.

Necropsy and analytical procedures. Animals were fasted (with free access to tap water) beginning at 4 pm the day before necropsy. They were anesthetized with sodium pentobarbital at approximately 50 mg/kg body weight. The colon was removed by blunt and sharp dissection, and the diaphragm was cut to prevent the animal from reviving postanesthesia. The contents of the

abdominal and thoracic cavities were examined for any grossly evident lesions.

The colon was slit open and feces were removed. The colon was washed by swirling in three successive beakers of ice-cold phosphate-buffered saline (PBS), pH 8.0. It was then spread flat on Parafilm and the length measured to the nearest 0.5 cm with a ruler. A segment at the midpoint of the colon measuring approximately 0.5 cm in length was removed and preserved in neutral buffered formalin (pH 7.4) for later mucin histochemical staining. The mucosa of the distal 12 cm of colon (the area of the majority of colonic neoplasms in previous reported studies (12, 18–20), as confirmed by personal communication with the author in the case of Ref. (20)) was then scraped off with an ice-cold glass microscope slide. The mucosa was weighed to the nearest 0.01 g and homogenized in 2 ml of pH 8.0 PBS with 10 strokes of a Ten Broeck tissue grinder immersed in ice. This homogenate was centrifuged at 159,000*g* at 4°C for 60 min. The supernatant was assayed for protein content by the Bio-Rad assay method (Bio-Rad Laboratories, Richmond, CA). After the protein assay, thymidine kinase activity of the supernatant was determined by the method of Klemperer and Haynes (21), using the slope of a plot of the formation of thymidine phosphate at 5-min intervals for 20 min. Analyses were conducted in duplicate for each incubation time, and thymidine kinase specific activities (μmole thymidine phosphate formed $\times 10^6$/min/mg protein) were calculated for each animal.

Mucin histochemical staining was done on the midcolon tissue specimens after dehydration and embedding in paraffin. Sections of 5–6 μm were cut and stained by the high-iron diamine/Alcian blue method of Spicer (9). These slides were identified by code numbers to allow the investigator to read them without knowledge of dietary treatment. Three well-oriented crypts were examined on each slide (a mean of 106 stained goblet cells per animal), and mucin-containing cells were classified on the basis of characteristic staining colors as sulfomucin predominant, sialomucin predominant, or mixed. After all slides had been read, the code was broken and the number of cells in each mucin category was expressed as a percentage of the total number of cells counted for each animal.

Statistical analysis. Body weights (Week 0 and Week 4), animal weekly food intakes, thymidine kinase specific activities, and mucin histochemistry percentage values were tabulated for each dietary group, and means and standard errors were calculated. Significant differences between groups were determined by the Kruskall–Wallis test, followed by Student-Newman–Keuls testing by ranks. Results were considered statistically significant at $\alpha < 0.05$ or lower.

Results. All animals appeared to be in good general health throughout the study. Mean body weights (±SE) at the start of the experiment ranged from 211.8 ± 8.3 to 215.5 ± 9.9 g. At the conclusion of the 4-week feeding period, mean weights of the rats in the four dietary groups ranged from 232.0 ± 9.1 to 253.4 ± 7.8 g. These minor weight differences were not statistically significant.

Food intake data showed minor variations between groups in the first 2 weeks of feeding, after which these differences disappeared. During the initial 2 weeks of feeding, mean (±SEM) caloric intakes ranged from 59.8 ± 2.3 to 76.0 ± 4.7 kcal/day. Early trends toward decreased caloric intake in the guar gum group and increased intake in the wheat bran group lost statistical significance during the last 2 weeks of feeding, resulting in a much narrower range of mean food intakes (62.7 ± 3.4 to 69.6 ± 1.3 kcal/day) during this period.

At necropsy, three animals in the carrageenan group were noted to have slight congestion and erythema of the distal 4–5 cm of colon. There were no other visible abnormalities noted upon gross examination of the rats.

Thymidine kinase specific activities are shown in Table II. The thymidine kinase specific activity of the carrageenan group was considerably higher than the specific activities of the other groups. This difference was highly statistically significant ($\alpha < 0.001$). Mean (±SEM) protein values associated with these thymidine kinase specific activities were 3.14 ± 0.22, 2.94 ± 0.26, 3.11 ± 0.25, and 4.15 ± 0.73 mg total protein for the mucosal supernatants from the fiber-free, guar

TABLE II. COLONIC THYMIDINE KINASE SPECIFIC ACTIVITIES OF RATS FED DIFFERENT FIBER-CONTAINING DIETS (UNITS = μmole THYMIDINE PHOSPHATE FORMED × 10^6/min/mg PROTEIN)[a]

Dietary group	Thymidine kinase specific activity
Fiber-free	10.98 ± 1.50
5% Guar gum	9.11 ± 2.04
10% Wheat bran	7.41 ± 1.09
5% Carrageenan	41.84 ± 4.65*

[a] Values are means ± SE; N/group = 8.
* The carrageenan group differs significantly from the other groups, $\alpha < 0.001$.

gum, wheat bran, and carrageenan groups, respectively. These minor differences were not statistically significant, and obviously cannot account for the observed differences in thymidine kinase specific activity. The thymidine kinase specific activity of the three animals in the carrageenan group noted to have erythematous distal colonic mucosa did not differ significantly from that of the other animals in this group.

Mucin histochemical staining failed to reveal any significant differences between dietary groups (data not shown).

Discussion. A notable finding of the present study was the marked increase in colonic mucosal thymidine kinase activity induced by feeding undegraded carrageenan. This is presumably indicative of a substantial increase in colonic mucosal cell proliferation, paralleling carrageenan's reported effects as an enhancer of chemically induced colonic tumorigenesis. In a study of rats fed semipurified diets and treated with the chemical carcinogen azoxymethane, ingestion of high levels of undegraded carrageenan resulted in a 100% tumor incidence versus only 57% for control animals, and perhaps more significantly a 10-fold greater number of tumors per rat (2). This is an unusual response among dietary fibers, and is reflected in the unusually high thymidine kinase activity detected in the present study. Determination of the long-term biological significance of this marked increase in thymidine kinase activity will require further study. It cannot be determined from a single short-term study such as the present one whether the observed effects are pathologic or merely transient and adaptive.

Various preparations of undegraded carrageenan may behave differently from one another *in vivo.* Glauert and Bennink (22) fed undegraded carrageenan at a 7.4% level to male Sprague–Dawley rats for 28 days. Autoradiographic examination of a segment of the distal colon revealed a 63% increase in the labeling index of the carrageenan-treated rats compared to the fiber-free controls. This difference did not achieve statistical significance, however, possibly secondary to the number of animals involved in the study. Personal communication with the authors of this study and the carrageenan suppliers revealed that the carrageenan used was a mixture of 80% κ- and 20% λ-carrageenans from *Chondrus crispus,* whereas our study employed carrageenan which was primarily of the λ-type, isolated from *Gigartina* species. These types of carrageenan differ dramatically in both gel strength and sulfate content, and thus differences in their physiologic effects might also be expected, on the basis of their differing chemical and physical properties. We were concerned that the carrageenan used in the present study be as similar as possible to that employed in the previously cited tumorigenesis study (2), hence our selection of the particular type of carrageenan used. Differing levels of cell proliferation induced by other types of carrageenan emphasize the usefulness of carefully defining the source and characteristics of dietary materials. Furthermore, results of this sort suggest that the simple term "carrageenan" may be an oversimplification in describing this diverse group of materials, much as the term "dietary fiber" may fail to fully connote the diversity of materials which are encompassed by such a general term.

Animals fed guar gum and wheat bran had colonic thymidine kinase activities similar to those of control animals. This is to be expected with wheat bran, as this fiber tends in general to reduce the incidence of chemically induced tumors, except in one report where bran was fed at high levels during carcinogen administration (23), or where very high doses of carcinogen were used. Therefore wheat bran would not be expected to increase cell proliferation, except perhaps at very high

levels in the diet (23). On the basis of the published literature available to the investigator at the time of the design of the study, it was expected that guar gum would produce an elevated level of colonic thymidine kinase activity, paralleling the increase in the yield of chemically induced tumors seen in a recently published study (6). Work by the same group also showed an increased labeling index in distal colonic cells of rats fed guar gum (24). These studies differed from the present one in that 10% rather than 5% guar gum was employed in the diets, and Sprague–Dawley rats were used rather than Fischer 344 rats. The reported enhancement of tumorigenesis by guar gum was statistically significant only when the incidence of adenocarcinomas was considered separately from benign tumors. When total tumor incidence was calculated, there was no significant difference between the guar gum and the fiber-free groups (6). Since completion of the present study, we have also learned of recent data showing lack of enhancement of chemically induced tumorigenesis by guar gum feeding at 5, 10, or 20% levels in Fischer rats (20), as well as another study showing no significant enhancement in colonic cell proliferation resulting from guar gum feeding (25). Therefore it appears that guar gum may be a relatively weak enhancer of tumorigenesis or may in fact lack such activity. Nor does stimulation of colonic cell proliferation appear to occur consistently with guar gum feeding, perhaps being affected by differences in rat strains or other components present in the diet. Differences in dose level may be particularly important with a fermentable fiber such as guar gum, with higher doses possibly resulting in greater amounts of fiber actually reaching the colonic tissues distal to the cecum. Unless a sufficient quantity of fiber actually reaches the colon, cell proliferation may not be affected. These considerations may explain the lack of observable increase in thymidine kinase activity after guar gum feeding found in the present study.

There was no significant difference in the distribution of mucin types between dietary groups as detected by high-iron diamine/Alcian blue staining. A number of investigators examining tissue specimens from patients with various colonic diseases have reported an increase in the amount of sialomucin relative to sulfomucin with increasing histologic dysplasia (26), or at the margin of human tumors found likely to recur (27). An increased proportion of sialomucin has also been found in otherwise histologically normal-appearing mucosa of rats treated with azoxymethane (10). On the other hand, some investigators have found an increased absolute number of sialomucin staining cells in axoxymethane-treated rats, but no significant change in the relative proportion of mucin types in these cells (28). Still others have reported no clear relationship between the type of mucin present and the degree of histologic dysplasia (29) or correlation with transformation by chemical carcinogens (30). There also exists considerable controversy among pathologists regarding the exact staining conditions required for the high-iron diamine/Alcian blue method, with consequent difficulty in reproducing results between laboratories (31). The lack of detectable fiber-induced alteration in mucin histochemistry in the present study may be due to these methodologic problems, or possibly because the dietary fibers employed were considerably weaker stimuli to cellular dysplasia than the chemical carcinogens or preexisting neoplasms used in other studies.

Measurement of colonic thymidine kinase activity is only one potential correlate of changes in cellular metabolism which may precede neoplasia. Numerous methods examining other aspects of cellular function possibly associated with preneoplastic changes have been reported. Most methods were developed using tissues of cancer patients, premalignant tissues, or animals treated with chemical carcinogens, and therefore probably represent indicators of both initiation and promotion, without clearly differentiating the two. Such methods include measurement of tissue lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities (32), mucosal binding of *Ulex europeus* agglutinin (30), changes in tissue glycosphingolipid composition (33), and presence of *ras* oncogene transcripts (34), among others. Methods intended primarily to examine the promotion process generally rely on markers of cell proliferation, such as incorporation of [^{3}H]thymidine

as assessed by autoradiography (24), tissue levels of ornithine decarboxylase enzyme activity (35), isolation of labeled cellular DNA after [^{3}H]thymidine administration (24), or detection of cells undergoing mitosis, using a metaphase arrest technique (25). Autoradiography is perhaps the "gold standard" of measurement techniques, but it is both time consuming and tedious, requiring the counting of labeled cells in as many as 40 crypts per sample to achieve highly reproducible results (36). Measurement of ^{3}H activity in isolated mucosal DNA after *in vivo* [^{3}H]thymidine administration eliminates the need for manual counting of labeled cells. However, examination of the data from the study in which this method was used (24) indicates that autoradiography is a more sensitive measure of proliferative changes in the distal colon. Ornithine decarboxylase enzyme activity seems to correlate positively with abnormal proliferative states in the hands of some investigators (35), while others have found an inverse correlation (37) or noted similar levels in normal and carcinomatous lesions (38). Further standardization of this method appears to be necessary to allow achievement of consistent results in different laboratories. Detection of proliferating cells with a metaphase arrest technique (16, 25) eliminates the need for *in vivo* radioisotope administration, although counting of individual stained cells and the sequential killing of several rats appear to be necessary to produce a single value reflecting cell proliferation.

Thymidine kinase activity as measured in the present study may represent a useful indicator of colonic cell proliferation. Previous reports have shown that thymidine kinase activity is elevated in the small intestinal mucosa by feeding some types of dietary fiber (8), and is increased in colon tumors compared to normal mucosa (3). The technique is straightforward and the colonic specific activity of a set of four animals can be determined in an afternoon. Because of its ease and rapidity of determination, as well as its evident responsiveness to alterations in dietary fiber intake, colonic thymidine kinase would seem to be a useful marker of fiber-induced colonic cell proliferation.

The present study has shown that colonic mucosal thymidine kinase activity is useful in detecting changes in cell proliferation induced by dietary fibers reported to increase the yield of chemically induced colon tumors. Fibers reported to have equivocal or no effects on cancer promotion show no detectable alteration in thymidine kinase activity. The high-iron diamine/Alcian blue method as described by Spicer (9) does not appear to be sensitive enough to allow detection of differential effects of various dietary fibers.

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