Protective Effects of Hard Red Versus Hard White Winter Wheats in Chemically Induced Colon Cancer in CF1 Mice[1]

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ABSTRACT

The relative protective effects of hard red versus hard white winter wheats in 1,2-dimethylhydrazine-induced colon cancer in CF1 mice were compared, and the effects of both whole wheats were compared with that of cellulose. Animals (50 per group) fed red wheat diets containing the whole wheat, bran, or endosperm had lower incidence of tumor development than those fed respective white wheat diets. Tumor incidence in animals fed a synthetic diet containing cellulose was not different from those fed the whole wheat diets containing similar amounts of insoluble dietary fiber. However, the number of tumors per tumor-bearing animal (multiplicity) was significantly lower in animals fed red whole wheat than it was in those fed the cellulose diet. Only animals fed the red bran diet had tumor incidence lower than that of the cellulose-fed animals. The tumors were located in the lower one-third of the colon; no metastasis to local lymph nodes and other tissues had occurred. Colon tumor incidence was weakly and inversely correlated with insoluble dietary fiber ($r = -0.2477$, $P = 0.1050$), total dietary fiber ($r = -0.2489$, $P = 0.1033$), and phytic acid ($r = -0.2327$, $P = 0.1285$) in the diets. Total phenolic compounds and vitamin E were higher in the red wheat than they were in the white wheat. The total amount of fecal neutral sterols excreted per day was significantly higher for animals fed red bran diet than it was for animals fed any other diet. There were no significant differences in excreted bile acid amounts between animals fed any white versus red wheat diets. Data suggest that no single factor measured plays a major role in protecting against chemically induced colon cancer in mice, but all of those factors may exert some antitumor effect.

It is generally agreed that some types of dietary fiber, especially insoluble fiber, can affect colon cancer incidence (Roberfroid 1993). Many, but not all, studies have shown that fiber-rich wheat bran can protect against chemically induced colon cancer in experimental animals. When the overall findings of a large number of studies were compared, wheat bran appeared to have a protective effect in 13 of the 17 studies. However, it actually increased the number of tumors in one study (Pilch 1987). Few of those studies identified the source of the bran. In addition, hardly any information was given as to what milling fraction constituted the "bran". Differences in those factors could have contributed to the variability in results. Cellulose, which comprises about 18% of the fiber in wheat bran (Holland et al 1988), also has been shown to protect against chemically induced colon cancer (Pilch 1987).

It has been suggested that some nonfibrous components of fiber-rich foods might be responsible for reported protective effects. Phytic acid, which is present in all cereals and is highly concentrated in the bran, has been shown to complex with iron, preventing hydroxyl radical formation and lipid peroxidation, events thought to be important in the process of tumor development (Graf 1986). According to Graf and Eaton (1985), dietary phytate, rather than fiber per se, might be the most important variable governing the frequency of colonic cancer. Pool-Zobel et al (1993) have recently reported antigenotoxic effects of phytate in the rat gastrointestinal tract.

Other possible anticancer components of grain-rich foods are the relatively high levels of phenolic compounds and vitamin E. Wattenberg et al (1980) found that feeding high levels of phenolic acids suppressed benzo(a)pyrene-induced neoplasm of the forestomach in mice. Cook and McNamara (1980) reported that significantly fewer adenomas and invasive carcinomas occurred in 1,2-dimethylhydrazine (DMH)-challenged mice fed higher dietary levels of vitamin E.

One way in which dietary fibers are thought to inhibit colon carcinogenesis is by reducing the concentration of bile acids or neutral sterols in the colon. Bile acids have been shown to act as tumor promoters in colon cancer development (Story and Furumoto 1990). Colon bacteria can convert primary bile acids into smaller, secondary, bile acids that may increase the risk of colon cancer by increasing the turnover of intestinal mucosal cells. Insoluble dietary fiber may dilute the concentration of secondary bile acids in the colon and, therefore, be protective against colon cancer (Slavin 1990).

The major objective of this investigation was to compare the cancer-preventing potential of hard white winter wheats to that of hard red winter wheats in DMH-treated mice. We also wanted to compare the cancer-preventing effects of whole wheat diets with those of a synthetic diet containing cellulose and determine whether dietary factors other than fiber (e.g., phytic acid, phenolic compounds, or vitamin E) might play a role.

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MATERIALS AND METHODS

Materials

Hard red winter wheat [Norkan (88-854)] and hard white winter wheat [KS84HW196 (88-850)] grown in 1988 at Fort Hays Agricultural Experiment Station, Hays, KS, were obtained. Each wheat cultivar was milled to whole wheat flour or straight grade flour of 74% extraction, and bran (26% of grain). Whole wheat flour was milled using a stone mill (Europemill EM-25/251, Denmark). The straight grade flour and bran were milled using the Miag Multomat S/100 (Braunchweig, Germany). After being milled, fractions were stored in a cold room (2°C) until needed for research. Bran particle size was reduced using a Fitz mill Model D-comminutor with a 1/16-in. (1.5-mm) screen (Fitzpatrick Co., Muncie, IN). Diets were mixed in a Wenger ribbon mixer (Wenger Manufacturing Co., Sabetha, KS).

Diet Preparation

Seven dietary treatments included: 1) casein-base with alphacel cellulose (control), 2) hard white wheat whole flour, 3) hard red wheat whole flour, 4) hard white wheat bran, 5) hard red wheat bran, 6) hard white wheat straight grade flour, or 7) hard red wheat straight grade flour (Table I).

Diets were formulated to contain 20% protein, 6% fat, and 45 or 50% by weight of the test product. Originally, we had planned to test each wheat component at the 50% level in the diets. However, preliminary work showed that the mice fed 50% bran diets did not thrive at that high fiber level, so concentration of test product in the those diets was dropped back to 45%, and the other dietary components were adjusted accordingly to keep the bran diets isonitrogenous with the other test diets. Protein (N × 6.25), fat, ash, calculated caloric value, insoluble dietary fiber (IDF), soluble dietary fiber (SDF), and total dietary fiber (TDF) contents of the diets are shown in Table II. Standard AACC (1983) methods were used: method 46-16 for protein; method 30-25 for fat; method 08-01 for ash; and method 32-07 for IDF, SDF, and TDF.

The method of Tangkongchitr et al (1981) was used to determine phytic acid in wheat whole flours, brans, and straight grade flours (Table II). As expected, the bran diets had the highest concentrations of phytic acid. The straight grade flour diets had the lowest concentrations of phytic acid, and the whole flour diets had intermediate concentrations. Phytic acid concentration in respective red versus white wheat diets was not significantly different. Phytic acid was highly correlated with insoluble dietary fiber ($r = 0.9579$, $P = 0.0001$) and total dietary fiber ($r = 0.9819$, $P = 0.0001$). Total phenolic compounds were extracted from the grains by the method of Pussayanawin and Wetzel (1987). They were then measured colorimetrically (Swain and Hillis 1959).

Vitamin E in the whole wheat flours and brans was extracted and separated using high-performance liquid chromatography by the method of Cort et al (1983). After the extraction and separation, vitamin E was determined fluorometrically (Speek et al 1985).

TABLE I
Percent Composition of Mouse Diets

Diet[a]	Cereal	Casein[b]	Corn Starch[b]	Fat[c]	Cellulose[d]
Control (with cellulose)	0	23.5	47.0	5.0	6.0
White whole flour	50	14.9	12.4	4.2	...
Red whole flour	50	13.4	14.0	4.1	...
White bran	45	12.9	20.4	3.2	...
Red bran	45	12.0	21.2	3.3	...
White straight grade flour	50	14.7	12.2	4.6	...
Red straight grade flour	50	14.9	12.1	4.5	...

[a]All diets contained 4% salt mixture XVII, 1% vitamin mix 2 (both obtained from ICN Nutritional Biochemicals, Cleveland, OH), 1% cholesterol, 0.30% *dl*-methionine, 0.2% choline bitartrate (all from Sigma Chemical Co., St. Louis, MO), and 12% sucrose.
[b]From Sigma.
[c]Vegetable oil (soybean) from a local supermarket.
[d]Alphacel from ICN.

Cancer Study

Seven groups of 50 CF1 mice (Charles River Breeding Laboratory, Wilmington, MA), initially weighing 28 ± 1 g, were housed in an environmentally controlled room with a 12-hr light-dark cycle. Food and water were provided ad libitum. Animals were weighed weekly. Animals received subcutaneous injections of DMH (Aldrich Chemical Co., Milwaukee, WI) once weekly for 20 weeks at a dosage of 20 mg/kg of body weight (Glauert et al 1981). When DMH is given in this way, the incidence and number of tumors per tumor-bearing animal tends to be reduced, which maximizes the sensitivity of the method to finding differences caused by diet (Aruoma 1993). The DMH was prepared fresh by dissolving 0.2 g in 100 ml of physiological saline solution (Glauert et al 1981). The drug has been found by several investigators to have carcinogenic specificity for the colon. According to Weisburger (1971), DMH is metabolized in the liver, and its complex chemical breakdown products exit through the bile into the lower part of the intestinal tract, where the active carcinogenic agent is released by bacterial action.

Half of the animals from each group were sacrificed after 20 weeks (end of initiation period) by exposure to an ether atmosphere. The remainder of the animals were fed the experimental diets for another 20 weeks (promotion period), then surviving animals were sacrificed in the same way. Necropsies were performed on animals that did not survive the full 40 weeks to determine cause of death.

Feces were collected daily during weeks 39 and 40. After each collection, feces were sealed in plastic freezer bags and stored frozen until analyzed. Total neutral sterols and bile acids were extracted with absolute ethanol from oven-dried and finely ground fecal samples by the method of Roscoe and Fahrenbach (1963). An aliquot of the extract was saponified, and neutral sterols were removed by extracting the saponified, dried extract with hexane. Bile acids were extracted from the residue with chloroform after removal of neutral sterols. The chloroform was evaporated, and acidic pigments were removed by redissolving the residue in a solution of benzene and methanol (1:1, v/v), and activated charcoal was added. The mixture was then filtered through Whatman No. 1 paper. The filtrate was evaporated to dryness under nitrogen. The residue was extracted with hexane to remove free fatty acids. The extracted residue was dissolved in 0.1*N* NaOH. Total bile acids were determined colorimetrically (Collings et al 1979).

The entire cecum and colon with rectum and all organs of

TABLE II
Percentage of Protein, Fat, Ash, Dietary Fiber,[a] Phytic Acid, and Calculated Caloric Content of Mouse Diets (as Fed)

Diet	Protein	Fat	Ash	Dietary Fiber			Phytic Acid	Energy (kcal/100 g)
				IF	SF	TDF		
Control (with cellulose)	20.9	6.5	3.21	4.75	0.00	4.75	0.01	384
White wheat whole flour	20.8	6.4	5.64	5.90	1.56	7.46	0.48	393
Red wheat whole flour	20.4	6.0	5.90	5.82	1.44	7.26	0.45	393
White wheat bran	21.3	7.3	5.42	16.16	2.56	18.72	1.52	325
Red wheat bran	21.3	7.3	5.91	17.36	3.09	20.45	1.58	325
White straight grade flour	19.9	6.7	4.01	0.61	1.02	1.63	0.09	406
Red straight grade flour	21.1	6.7	4.60	0.65	0.97	1.62	0.08	406

[a]IF = insoluble dietary fiber, SF = soluble dietary fiber, TDF = total dietary fiber.

the thoracic and abdominal cavity were taken for histopathological examination. The colon was divided into three equal parts (upper, middle, and lower colon). The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin-eosin (H&E), then processed in an Autotechnicon before histopathological examination. Selected tissues were further stained by the periodic acid-Schiff (PAS) method (Ward 1974). The number of animals with tumors per group, the number of tumors per tumor-bearing animal, and tumor distribution and type were evaluated on blind samples.

Data were analyzed by the Statistical Analysis System (SAS 1989) using one-way analysis of variance with Fisher's protected least significant difference (LSD) test for significant differences among means (Ott 1988).

RESULTS AND DISCUSSION

Effect of Wheat Diets on Mouse Weight Gains

At the end of the initiation period (20 weeks), animals fed respective hard white wheat and hard red wheat diets had similar overall weight gains (Table III). Animals fed the whole wheat flour diets had the highest overall weight gains; animals fed bran and straight grade flour diets had similar overall weight gains. Mice fed the diet containing no cereal product (control) had the lowest weight gains.

By the end of the promotion period (40 weeks), significant differences were still not observed between animals fed respective white or red wheat diets. At that time, only animals fed the whole flour diets had gained significantly more weight than had animals fed the control diet (Table III). No significant relationships were observed between overall weight gains (after 40 weeks) and insoluble dietary fiber, soluble dietary fiber, and total dietary fiber.

Colon Tumor Incidence

At the end of the initiation period (20 weeks), only animals fed control, white wheat bran, and white straight grade flour diets had developed tumors (18, 9, and 5% respectively), but the differences were not significant (Table IV). No animals fed hard red wheat fractions had developed colon tumors.

After the promotion period (40 weeks), animals fed the red wheat whole flour, bran, or straight grade flour diets had lower incidences of tumor development than had animals fed the corresponding white wheat diets (Table IV). Tumor incidence was not different in animals fed the whole wheat diets and the cellulose diet. That is in contrast to reports by Watanabe et al (1979) and Reddy and Mori (1981), who found that rats treated with DMH and fed a diet containing wheat bran had lower incidence of colon tumor development than did those fed a basal diet containing cellulose as a fiber source. Only animals fed the red bran diet had a significantly lower incidence of tumor development than that of animals fed the cellulose diet. No group had a statistically higher incidence of tumor development than those fed the cellulose diet without cereal product.

TABLE III
Mouse Weight Gains (g) After Initiation and Promotion Periods[a]

Diet	Initiation (20 weeks)	Promotion (40 weeks)
Control (with cellulose)	39.8 d	43.5 cd
White whole flour	46.2 a	47.0 ab
Red whole flour	44.9 a	48.3 a
White bran	41.3 bc	44.8 b–d
Red bran	40.3 cd	42.3 d
White straight grade flour	42.3 b	45.4 a–c
Red straight grade flour	40.9 b–d	43.2 cd
LSD	1.47	3.05

[a] Means in the same column not followed by the same letter are significantly different ($P < 0.05$).

Multiplicity (Number of Tumors per Tumor-Bearing Animal)

At the end of the promotion period, multiplicity was not significantly different between animals fed white or red wheat diets (Table IV). Animals fed the cellulose diet had the highest multiplicity, but the difference was significant only for groups fed red whole flour and white bran diets. Reddy and Mori (1981) also reported that multiplicity was lower in animals fed a diet containing wheat bran than in those fed a diet containing cellulose. Clapp et al (1984) reported that multiplicity ranged from 1.4 to 1.6 after mice were fed a semisynthetic diet containing 20% added bran (either corn, soybean, soft winter wheat, or hard spring wheat). In this study, multiplicity ranged from 1.33 to 2.70.

Tumors were found much more commonly in the lower colon but also were observed in the middle colon (Table IV). Although Cruse et al (1982) reported that cholesterol feeding of rats with colon cancer induced by DMH resulted in increased metastasis, no metastasis to local lymph nodes and other tissues occurred in the present study, in which the mice consumed 1% dietary cholesterol. Only animals fed the cellulose containing diet had tumors in the upper colon.

Tumor Type

All benign colon tumors were polyploid neoplasms or adenocarcinomas and were confined to the epithelial layer (carcinoma in situ). Others have reported similar tumors in DMH-treated animals (Freeman et al 1978). The tumors began in the mucosa, grew into the lumen, and were noninvasive as shown in Figure 1. By comparison, Figure 2 shows a normal mouse colon.

Other Clinical Observations

Survival rate was lowest (61%) for animals fed the red straight grade flour; survival rate of control animals was 82% and ranged from 82 to 91% in all other groups (Table IV). However, of the 31 animals that died during the promotion period, only two had colon tumors. The destructive effect of DMH was obvious, with a number of the animals having rectal bleeding and some grossly observable destructive lesions in the perianal region. Among the animals that died before sacrifice, presumptive causes of death included squamous cell carcinoma of the ear (one animal fed red bran diet), hemangiosarcoma (blood vessel tumor), and retroperitoneal cancer with metastasis to kidney. (Nine animals fed brans and straight grade flours developed kidney tumors.) Hepa-

TABLE IV
Survival Rate (%), Percent of Mice With Tumors After Initiation and Promotion Periods, Multiplicity, and Tumor Distribution in Colon[a]

Diet	Survival Rate	Percent with Tumors		Multiplicity	Percent Tumor Distribution		
		Initiation (20 weeks)	Promotion (40 weeks)		Lower Colon	Middle Colon	Upper Colon
Control	82	18	60.4 a–c	2.70 a	90	5	5
White whole flour	88	0	72.2 ab	1.90 a–c	90	10	0
Red whole flour	91	0	51.4 cd	1.33 c	78	22	0
White bran	87	9	59.6 a–c	1.55 bc	91	9	0
Red bran	83	0	37.8 d	2.44 ab	89	11	0
White straight grade flour	82	5	73.6 a	1.85 a–c	85	15	0
Red straight grade flour	61	0	50.0 cd	1.83 a–c	83	17	0
LSD			21.5	1.09			

[a] Means in the same column not followed by the same letter are significantly different ($P < 0.10$).

tocytes had vacuolated cytoplasm, and some animals developed liver tumors (animals fed red bran and red straight grade flour diets). Four animals fed control and red wheat fraction diets had lung tumors.

Role of Dietary Phytic Acid, Phenolic Compounds, and Vitamin E

There was a weak negative correlation between dietary phytic acid content and tumor incidence ($r = -0.2327$, $P = 0.1285$). However, phytic acid contents of the red and white wheats were similar (Table II), and it is unlikely that phytic acid was responsible for the lower cancer incidence in animals fed the different whole wheats or wheat fractions.

Total phenolic acid concentration was somewhat higher in the red whole wheat (104 μg/g of sample) than in the white wheat (93 μg/g of sample). Fractionation and quantitation of individual phenolic compounds in the wheats might provide insight into the relative anticancer effects of the two wheats.

The observation that tumor incidence was not different in animals fed the cellulose or wheat whole flour diets reinforces the conclusion that phytic acid and phenolic compounds did not play a major role in preventing colon cancer, because the cellulose diet contained essentially no phytic acid or phenolics.

Vitamin E content of both whole wheat flours was less than 0.500 mg/100 g of sample; it could not be measured more accurately because of the limitations of the method used (Speek et al 1985). However, the red wheat bran did contain higher concentration of vitamin E than did the white bran (2.44 mg/100 g of sample vs. 1.73 mg/100 g of sample). Animals fed the red wheat bran diet had a significantly lower incidence of tumors than did animals fed any other diet; higher vitamin E levels in that diet might have contributed to its protective effect. More sensitive assays of vitamin E content of the various fractions should be performed.

Total Fecal Neutral Sterols

No significant differences occurred in fecal neutral sterol concentrations for animals fed hard white versus those fed hard red winter wheats (Table V). Among animals fed the different wheat diets (whole flours, brans, straight grade flours), those fed bran diets had the lowest concentration of total neutral sterols in feces, and those fed straight grade flour diets had the highest neutral sterol concentrations. Animals fed the whole flour diets had intermediate levels, as did the control animals.

The total amount of fecal neutral sterols excreted per day was significantly higher for animals fed the red bran diet than for those fed any other diet (Table V). Fecal neutral sterol concentration was inversely correlated to insoluble dietary fiber ($r = -0.8382$, $P = 0.0001$) and total dietary fiber ($r = -0.8204$, $P = 0.0001$). The amount of sterols excreted daily in feces was positively correlated with insoluble dietary fiber ($r = 0.5667$, $P = 0.0001$), and total dietary fiber ($r = 0.5789$, $P = 0.0001$). No significant relationship was observed between concentration of neutral sterols in feces and tumor incidence ($r = 0.1545$, $P = 0.3167$), but the amount of neutral sterols excreted daily was significantly correlated with tumor incidence ($r = -0.3385$, $P = 0.0540$). The results suggest that daily loss of neutral sterols in the feces may play a role in protecting against colon tumor development.

Total Fecal Bile Acids

No significant differences occurred in fecal bile acid concentrations and daily amounts excreted by mice fed red wheat diets versus those fed white wheat diets (Table V). Bile acid concentration and daily amounts also were not different for animals fed the red or white whole wheat diets or the cellulose diet. Feces of animals fed bran diets had the lowest bile acid concentrations and those fed straight grade flours had the highest concentrations of bile acids. Feces of animals fed whole wheats had intermediate

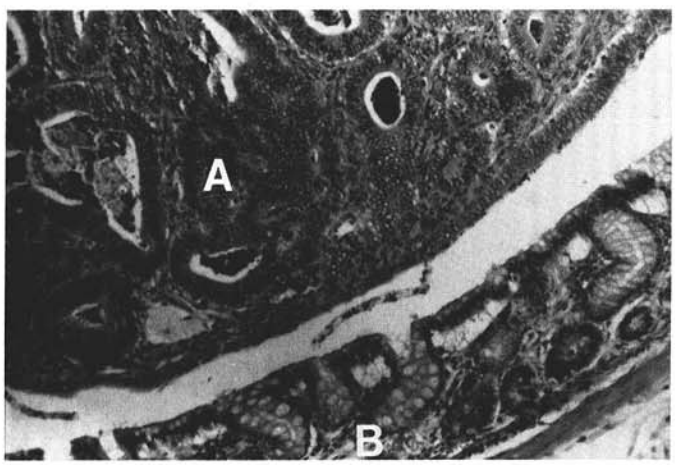

Fig. 1. Cross-section of a mouse colon with a tumor (A) in the lumen of the colon. Mouse was fed straight grade flour. Notice compression of mucosa (B) of colon due to tumor.

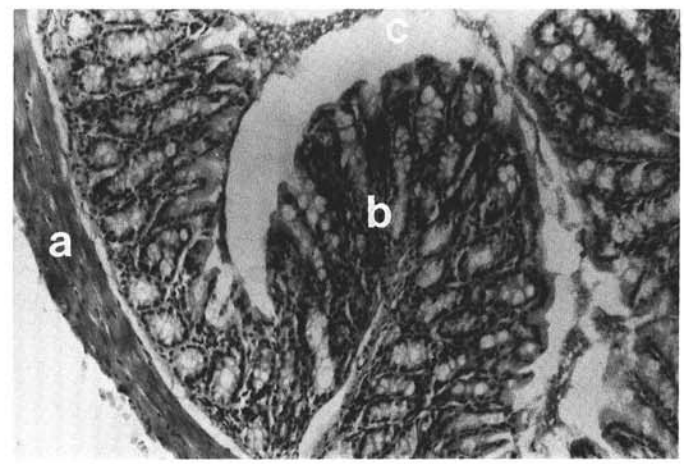

Fig. 2. Cross-section of a normal mouse colon from mouse fed control diet. Note the mucosal folds and regular arrangement of mucosal structures: muscular layer (a); colonic mucosa (b); lumen (c).

TABLE V
Effects of Wheat Diets on Total Fecal Neutral Sterols and Bile Acids of Mice After Promotion Period[a,b]

Diet	Total Neutral Sterols		Total Bile Acids	
	(mg/g of sample)	(mg/day)	(mg/g of sample)	(mg/day)
Control	8.31 b	5.50 c	7.99 b	5.18 ab
White whole flour	8.05 b	6.30 bc	7.96 b	6.56 ab
Red whole flour	7.46 b	7.25 bc	7.95 b	7.50 a
White bran	4.64 c	7.31 b	4.25 c	7.33 a
Red bran	5.31 c	9.33 a	3.06 c	5.45 ab
White straight grade flour	13.44 a	5.79 bc	11.15 a	4.53 b
Red straight grade flour	14.13 a	5.86 bc	11.75 a	4.25 b
LSD	2.01	1.77	2.31	2.77

[a] Means in the same column not followed by the same letter are significantly different ($P < 0.05$).
[b] Dry matter basis.

bile acid concentrations, which were not different than those for feces of control animals.

According to the bile acid hypothesis, a correlation between tumor incidence and fecal bile acid concentration might have been expected, but no significant relationships with fecal bile acid concentration or daily amount excreted in the feces were observed in this experiment. However, animals with the lowest tumor incidence (those fed red bran) also had the lowest fecal bile acid concentration.

CONCLUSIONS

A significant difference in anticancer activity was observed between the red and white wheats, with animals fed the red wheat having lower tumor incidence than that of animals fed the corresponding white wheat diets with similar total dietary fiber, fat, and calorie contents. However, correlation analyses showed weak inverse relationships between tumor incidence and insoluble dietary fiber ($r = -0.2477$, $P = 0.1050$) and total dietary fiber ($r = -0.2489$, $P = 0.1033$). Phytic acid content of the two wheats also was similar, and the correlation between dietary phytic acid and tumor incidence was not significant ($r = -0.2327$, $P = 0.1285$). Phenolic compounds and vitamin E were higher in the red than than they were in the white wheat, and they might have contributed to the protective effect. When diets contained similar levels of insoluble dietary fiber from red or white whole wheat or cellulose, their protective effects were not different, despite the fact that the cellulose diet contained essentially no phenolic compounds or phytic acid, and less vitamin E than other diets. High daily excretion of neutral sterols, but not bile acids, was correlated with reduced colon cancer incidence ($r = -0.3385$, $P = 0.0540$). Specific complex polysaccharides or lignin contents of the dietary fiber residues were not measured in this experiment, but it is possible that differences in those constituents of the wheats might affect their anticancer activity.

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