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Effect of Resistant Starch and/or Fat-Soluble Vitamins A and E on the Initiation Stage of Aberrant Crypts in Rat Colon

Sylvie Mazière, Khaled Meflah, Emmanuelle Tavan, Martine Champ,
Jean-Francois Narbonne, and Pierrette Cassand

Abstract: This study reports the modulating effects of resistant starch (RS) and the fat-soluble vitamins A or E, alone or in combination, on initiation of preneoplastic lesions in rat colon aberrant crypt foci (ACF) induced by 1,2-dimethylhydrazine. One group of male Sprague-Dawley rats was fed a basic diet and five groups were fed experimental diets supplemented with 25% RS, 200 IU vitamin A, 5 IU vitamin E, 25% RS + 200 IU vitamin A, or 25% RS + 5 IU vitamin E for four weeks. After induction by 1,2-dimethylhydrazine, all the animals were fed basic diets for four more weeks before sacrifice. Compared with the basic diet, only the vitamin A-supplemented diet significantly reduced the incidence of ACF. The vitamins incorporated in the animals' diets increased the vitamin concentrations in hepatic and colonic cells compared with the animals fed the basic diet. The preventive effect of vitamin A seems to be due to a direct effect on colonic epithelial cells. The three diets supplemented with RS significantly decreased cecal pH and bacterial β -glucuronidase activity and increased cecal weight and fecal output. The retrograde high-amylose maize, type 3, used in this study does not significantly decrease ACF. This RS has an effect on the colon similar to that of nonstarch polysaccharides. Neither biochemistry nor four weeks of dietary supplementation is likely sufficient for adaptation of the rat colonic flora.

Introduction

Many epidemiological and experimental studies suggest that a high intake of fruits and vegetables may prevent large bowel carcinogenesis (1,2). Fruits and vegetables constitute a major source of fiber, and since Burkitt's observations (3), dietary fibers have been implicated in the protective effect of fruits and vegetables against colon cancer. However, factors in plant foods, in addition to fiber, may also be chemopreventive agents (2). A protective role of antioxidants, including

carotenoids, vitamin E, and vitamin C, has been observed in numerous epidemiological (4) and animal (5,6) studies. β -Carotene is the most abundant and the most converted of the provitamin A carotenoids (vitamin A precursors) in vegetables and fruits. Because of its recognized role in normal cell differentiation, the chemopreventive effect of vitamin A has often been studied. Several authors have shown a protective effect of retinoids on chemically induced colon cancer in animals (7-10), but others have not obtained these results (11). Some contradictory results were also noted for the protective effect of dietary fibers against colon cancer (12). Insoluble grain fibers appear more protective than soluble dietary fibers. Different hypotheses have been proposed to explain the protective effects of fibers against colon cancer (12): dilution (12) or binding of carcinogens (13) and promoting agents such as bile acids (14); decrease in their contact with colonic mucosa; modulation of colonic bacterial enzymes occurring in the generation of toxic and carcinogenic substances (15); influence on the xenobiotic-metabolizing enzymes, which play a role in chemical carcinogenesis (16,17); and production of short-chain fatty acids (SCFA), particularly butyrate, which slows proliferation and stimulates differentiation of colon cancer cells *in vitro* (18). Dietary fibers are physically and chemically heterogeneous; their fermentation degree varies with the type and the physical state of the fiber. It appears that slowly fermented fibers have a greater influence on the distal environment (19). This is particularly important, because colorectal cancer is more common in the distal large bowel, and in a chemically induced colon cancer, 95% of the tumors are located in the distal colon (20). The undigested starch, called resistant starch (RS), may have effects comparable to those of nonstarch polysaccharides on colonic functions (21). This has led to the speculation that starch reaching the colon may have important consequences for preventing colon cancer. Caderni and co-workers (22) showed, in high-fat-low-calcium-low-cellulose diets, that starch has a protective role against 1,2-dimethylhy-

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drazine (DMH)-induced colon carcinogenesis. We also previously showed a protective effect of RS, alone or combined with vitamin A, on the promotion of precursor lesions of colon cancer in rats (23). However, a recent study showed more frequent tumors and greater number of aberrant crypt foci (ACF) in rats fed a potato starch diet (24). Furthermore, the physiological consequences of RS reaching the colon are not clear.

The aim of this study was to examine the effects of RS and fat-soluble vitamins A and E, individually and when combined, on the initiation of DMH-induced colonic preneoplastic lesions in rats. In addition, we have examined the influence of these experimental diets on some physiological parameters (SCFA concentrations in colon and cecum, cecal pH, hepatic enzyme activities, fecal output, β -glucuronidase activity, hepatic and colonic vitamin concentrations) having possible modulating effects on colorectal tumorigenesis. The specific aim was to identify the dietary factors acting on the early stage of colon carcinogenesis and whether RS combined with high levels of fat-soluble vitamins in the same diet can offer further protection.

Materials and Methods

Animals

Male weanling Sprague-Dawley rats obtained from Charles River (St. Aubin les Elboeuf, Cléon, France) were housed singly with a 12:12-hour light-dark cycle at 50% humidity and $21 \pm 1^\circ\text{C}$. A total of 76 rats were randomly divided into 6 experimental groups and were given water ad libitum. The animals were weighed weekly throughout the study.

Diets

Each group of animals was fed one of six diets (Table 1). Each diet was obtained as a dry powder from the Institut National de Recherche Agronomique (Centre de Recherches de Jouy-en-Josas, domaine de Vilvert, Jouy-en-Josas, France). Vitamin and mineral mixes of the diets are used as described by Potier de Courcy and others (25). RS added to the basal diet partly replaced maize starch from the control diet. RS was obtained from the Society Eridania-Béghin Say and was an extruded retrograde high-amylose maize starch, type 3. All the experimental diets contain the same quantity of total starch (basic with 66% of maize starch alone and RS supplemented with 41% maize starch and 25% RS). The RS-supplemented diets are in fact 8% starch supplemented at the rat digestion level (2% from cellulose and 6% from the extruded retrograde starch). This 8% level of supplementation is high enough to produce a physiological effect but does not inhibit growth (26). The diets are isocaloric.

Protocol

The animals were separated into 6 groups: 5 experimental groups of 12 rats each and 1 basic group of 16 rats.

All the rats were fed experimental or basic diets for four weeks according to the design illustrated in Figure 1. At the end of this period, four rats from each experimental group and eight rats from the basic group were sacrificed by decapitation to study the effect of experimental diets in comparison with basic diet on physiological parameters.

One control rat in each group received two injections of the solvent (0.9% NaCl solution). The remaining animals in each group received two DMH injections (Fluka, l'Isle

Table 1. Composition of Basic and Experimental Diets^a

	Basic Diet	Experimental Diets				
		RS	Vitamin A	RS + Vitamin A	Vitamin E	RS + Vitamin E
Casein	18	18	18	18	18	18
Maize starch	66	41	66	41	66	41
Cellulose	2	2	2	2	2	2
Maize oil	2	2	2	2	2	2
Lard	6.3	6.3	6.3	6.3	6.3	6.3
DL-Methionine	0.2	0.2	0.2	0.2	0.2	0.2
Mineral mix ^b	4.5	4.5	4.5	4.5	4.5	4.5
Vitamin mix ^c	1	1	1	1	1	1
RS	0	25	0	25	0	25
Retinyl acetate, IU/g	0	0	200	200	0	0
α -Tocopherol, IU/g	0	0	0	0	5	5

a: Values are g/100 g diet dry wt, unless otherwise indicated. RS, resistant starch.

b: Mineral mix composition (per kg mineral mix): 380 g calcium monohydrogen phosphate, 240 g dipotassium phosphate, 180 g calcium carbonate, 69 g sodium chloride, 20 g magnesium hydroxide, 90 g magnesium sulfate, 8.6 g iron sulfate, 5 g zinc sulfate, 5 g manganese sulfate, 1 g copper sulfate, 20 mg cobalt carbonate, 40 mg potassium iodide, 20 mg ammonium molybdate, 20 mg sodium selenite, 800 mg sodium fluoride, and 500 mg chromium potassium sulfate dodecahydrate.

c: Vitamin mix composition (in mg/kg diet): 1,500 choline concentrate (50%), 100 vitamin E (500 IU/g), 10 vitamin A acetate (500,000 IU/g), 25 vitamin D₃ (100,000 IU/g), 45 niacin, 30 calcium pantothenate, 10 thiamin hydrochloride, 10 riboflavin, 10 pyroxidine hydrochloride, 100 ascorbic acid, 50 *p*-aminobenzoic acid, 2 folic acid, 13.5 vitamin B-12 concentrate (1 g/kg), 10 D-biotin, 1 menadione, 50 *meso*-inositol, and 8,033.5 sucrose.

Figure 1. Experimental design adopted for studying effect of experimental supplemented diets on physiological parameters and on initiation of aberrant crypt foci. DMH, 1,2-dimethylhydrazine; AC, aberrant crypts.

d'Abeau Chesnes, Saint Quentin Fallavier cedex, France) at 25 mg/kg body wt ip; the second injection was given three days after the first.

Three days after the last injection, all the animals were fed the basic diet. The animals were killed four weeks after the DMH injection period to study proliferation of aberrant crypts as described by Abdelali and collaborators (27).

Physiological Parameters

Measurement of cecal weight and cecal pH: Immediately after the rats were sacrificed, the cecum was removed and weighed with its luminal content, and the results were reported relative to body weight. The cecal pH was directly measured with a portable microprocessor pH meter (model HI 8424, Hanna Instruments).

Fecal sample bulk: Four weeks after the experimental diet was begun, feces were collected each 24 hours for four days from four randomly chosen rats in each group. To determine the fecal dry weights, fresh stools were weighed and placed in a drying oven at 45°C. Feces were weighed every day for four days until dry weights were stable.

SCFA Analysis

At the end of four weeks of the experimental diet, cecal and fecal samples were taken, dissolved in a 0.1% HgCl_2 solution, and stored at -80°C . For SCFA measurement, the cecal and fecal suspensions were thawed, a 1:10 dilution of 5% H_3PO_4 was added, and the suspension was centrifuged at 8,000 g for 10 minutes. The supernatants were analyzed by gas-liquid chromatography as previously described (28).

Preparation of Liver Microsomal and Cytosolic Fractions

Livers were removed, minced, and homogenized in three volumes of ice-cold 0.15 M KCl solution. The postmitochondrial fraction, obtained by centrifugation of the homogenate at 9,000 g for 20 minutes, was centrifuged for one

hour at 105,000 g. The cytosol was removed and frozen at -80°C . Final microsomal preparations were resuspended in 100 mM potassium phosphate buffer-20% glycerol (pH 7.4).

Protein levels in the microsomal and cytosolic preparations were determined by the method of Lowry and colleagues (29).

Measurement of Hepatic Enzyme Activities

Nitrosodimethylamine N-demethylase activity: Nitrosodimethylamine N-demethylase (NDMAde) activity was assayed using the method of Nash (30). The nitrosodimethylamine metabolism by cytochrome P-450 (CYP450) 2E1 leads to the formation of formaldehyde (31). Incubations contained a final volume of 1 ml of 0.1 M phosphate-6 mM MgCl_2 buffer (pH 6.8), microsomal suspension (volume containing 1 mg of microsomal proteins), and 5 mM nitrosodimethylamine. The reaction, occurring at 37°C for 10 minutes, was started by addition of 1 mM NADPH and stopped by 500 μl of 17% perchloric acid. After centrifugation at 2,000 g for 5 minutes, 1 ml of supernatant was mixed with 400 μl of Nash reagent and incubated at 70°C for 20 minutes. After cooling, the absorbance was measured at 412 nm. The controls contained all the constituents excluding nitrosodimethylamine and including dimethyl sulfide. The NDMAde activity was expressed in nanomoles of HCHO formed per minute per nanomole of CYP450.

Uridine diphosphoglucuronyl transferase activity: Uridine diphosphoglucuronyl transferase (UDPGT) activity was assayed using a modification of the method of Frei (32) with *p*-nitrophenol (PNP) as substrate. Incubations contained a final volume of 0.24 ml of 0.5 M tris(hydroxymethyl)amino-methane, 0.5 mM MgCl_2 buffer (pH 7.5), 1.041 mM uridine-diphosphoglucuronic acid, and microsomal suspension (volume containing 0.2–0.4 mg of microsomal protein). The reaction, occurring at 37°C for 10 minutes, was started by addition of 0.175 mM PNP and stopped by 0.6 ml of 0.2 N trichloroacetic acid. After centrifugation at 4,000 g for five

minutes, 0.6 ml of supernatant was mixed with 0.03 ml of 10 N potassium hydroxide. The absorbance at 400 nm was then determined. In the positive controls, the total amount of PNP was conjugated by the UDPGT previously denatured by trichloroacetic acid. The difference between positive control and the assays determines the level of PNP conjugated by the enzyme in each assay. The UDPGT activity was expressed in nanomoles of conjugated PNP per minute per milligram of microsomal proteins.

Bacterial β -Glucuronidase Activity

Bacterial β -glucuronidase activity was measured by the modified method of Goldin and Gorbach (33). Fresh cecal samples were suspended in 20 volumes of 0.1 M phosphate buffer (pH 6.4). The cecal suspensions were homogenized with an Ultra-Thurax homogenizer for 30 seconds at 13,500 rpm. Incubations were performed in triplicate at 37°C for 5 and 10 minutes in a shaking water bath. The final concentration of the incubation mixture included 0.1 M phosphate buffer (pH 6.4) and 1 mM PNP- β -glucuronide. The reaction was started by addition of 200 μ l of bacterial suspension and stopped with 1.5 ml of 0.25 M Na₂CO₃. After centrifugation at 3,000 g for 30 minutes, a reading of the supernatant was taken at 400 nm. The amount of PNP released was determined by comparison with a standard PNP curve. The cecal β -glucuronidase activity was expressed in nanomoles of released PNP per minute per milligram of cecal extract proteins.

Cecal extract protein determinations were done in duplicate following the method of Lowry and colleagues (29), with bovine serum albumin as the standard. To determine the correct value of protein, the measure was done directly on the cecal suspension and also after trichloroacetic acid addition in the latter for the precipitation of protein fraction. The value considered was the difference between the two values obtained.

Fat-Soluble Vitamin Measurement in Hepatic and Colonic Epithelial Cells

For four rats in each group, after the colons were removed and flushed, colonic epithelial cells were scraped and stored at -80°C; 1 mg of each liver was also stored at -80°C. Fat-soluble vitamin extraction was assayed using a modified method previously described (34). The colonic epithelial cells and pieces of liver were thawed and homogenized in a 100 mM phosphate buffer (pH 7.4). One volume of ethanol and 10 μ l of 2 mM retinyl acetate were added to the homogenate. Three volumes of hexane were added to the suspension, and after agitation for 15 minutes at 4°C in the dark the mixture was centrifuged at 4,000 g for 15 minutes. The hexane phase was removed, and this extraction step was started again. From colonic epithelial cells, 50% of the final hexane phase was evaporated and dissolved in 1 ml of ethanol. Only 1 ml of the hexane phase was evaporated from liver extract and then dissolved in 1 ml of ethanol.

The vitamin A concentration was determined by high-performance liquid chromatography (HPLC) in the conditions previously described (34).

The vitamin E quantity was also determined by HPLC and detected by fluorescence with an excitation wavelength at 295 nm and an emission wavelength at 340 nm.

Identification and Quantification of Aberrant Crypts

Immediately after the rats were sacrificed, the colons were removed and prepared according to Bird (35). The colons were flushed with 0.9% NaCl and slit open longitudinally from cecum to anus, placed between two pieces of filter paper, and fixed in 10% buffered formalin. Then the fixed colons were stained with 0.5% methylene blue to observe aberrant crypts (AC). The number of AC per colon was determined with use of a light microscope at $\times 40$ magnification. Crypts were considered aberrant if they were ≥ 2.5 times larger than the surrounding crypts.

Statistical Analysis

Experimental data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Differences with $p < 0.05$ were considered significant.

Results

Body Weights, Diets, Cecal Weight and pH, and Fecal Bulk

The body weight gains are reported in Table 2. We observed no significant differences among the six groups of animals. No significant differences were observed among amounts of diet ingested by each group of animals (data not shown). Cecal parameters (weight and pH) and fecal output were markedly affected by diets containing RS. RS in the diet caused an increase of cecal weight, more pronounced in the presence of vitamin E, a decrease of cecal pH, and an increase of fecal output. These differences, observed after four weeks on the diets supplemented with RS (1st sacrifice), disappeared after four weeks of basic diet (2nd sacrifice).

Quantification of AC

Figure 2 illustrates the effects of the different experimental diets on the mean number of AC per rat. No AC are observed in animals given 0.9% NaCl solution. Only the vitamin A diet significantly decreased the total number of AC in comparison with the basic diet (-33%). The vitamin E diet induced a greater number of AC per rat than the basic diet (+31%), but this increase is not statistically significant. The total number of AC per rat was slightly decreased in the three experimental groups supplemented with RS (-23%

Table 2. Cecal Weight and pH, Fecal Bulk, and Body Weight Gain^{a,b}

	Basic Diet (n = 4)	Experimental Diets				
		RS (n = 4)	Vitamin A (n = 4)	RS + Vitamin A (n = 4)	Vitamin E (n = 4)	RS + Vitamin E (n = 4)
Fecal bulk, g/24 h	1.26 ± 0.26	2.45 ± 0.19 [†]	1.31 ± 0.20	1.92 ± 0.51*	1.51 ± 0.1	2.30 ± 0.26 [†]
1st sacrifice						
	Basic Diet (n = 8)	RS (n = 4)	Vitamin A (n = 4)	RS + Vitamin A (n = 4)	Vitamin E (n = 4)	RS + Vitamin E (n = 4)
Body wt gain, g/4 wk	288 ± 33	281 ± 25	267 ± 29	257 ± 72	261 ± 26	192 ± 16
Cecal wt, g/100 g body wt	0.7 ± 0.1	1.7 ± 0.2*	0.9 ± 0.1	1.7 ± 0.5*	0.7 ± 0.1	2.9 ± 1.1 [†]
Cecal pH	6.4 ± 0.1	5.7 ± 0.6 [†]	6.4 ± 0.1	5.8 ± 0.1*	6.4 ± 0.1	5.4 ± 0.1 [†]
2nd sacrifice						
	Basic Diet (n = 8)	RS (n = 8)	Vitamin A (n = 8)	RS + Vitamin A (n = 8)	Vitamin E (n = 8)	RS + Vitamin E (n = 8)
Body wt gain, g/9 wk	384 ± 33	400 ± 50	369 ± 26	372 ± 38	389 ± 28	378 ± 30
Cecal wt, g/100 g body wt	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	1.1 ± 0.7	0.7 ± 0.2	1.1 ± 0.7
Cecal pH	6.6 ± 0.3	6.5 ± 0.1	6.5 ± 0.4	6.7 ± 0.2	6.6 ± 0.3	6.4 ± 0.2

a: Values are means ± SD; n, number of rats.

b: Statistical significance is as follows: *, significantly different from basic ($p < 0.05$); †, very significantly different from basic ($p < 0.01$), by analysis of variance and Tukey's test.

Figure 2. Effects of experimental diets on total number of AC in DMH-treated rats. RS, resistant starch. Vertical bars, SD. Analysis of variance and Tukey's test were used for statistical comparison: *, $p < 0.05$.

with RS diet, -22% with RS + vitamin A diet, and -16% with RS + vitamin E diet), but this is not statistically significant. The crypt multiplicity represented by AC/ACF and the number of small ACF (ACF < 2AC) or large ACF (ACF > 3AC) per rat according to Corpet and colleagues (36) were not modified in experimental groups in comparison with the control group (data not shown).

SCFA Analysis

The results of SCFA analysis are shown in Table 3. The acetate, propionate, and total SCFA concentrations de-

creased along the colon length. The butyrate concentration was unmodified between cecal and fecal contents.

The cecal concentrations of all SCFA were not significantly modified by the experimental diets, in comparison with the basic group. However, the RS + vitamin A-supplemented diet significantly increased the concentration of acetate in the cecum (+77%, $p < 0.05$).

The fecal concentration of all SCFA was not significantly modified by the experimental diets, in comparison with the basic group. However, the fecal concentration of propionate was significantly decreased in rats fed the RS + vitamin A-supplemented diet in comparison with group fed the basic diet (-77%, $p < 0.01$).

Table 3. SCFA Concentration^{a,b}

	Basic Diet (n = 8)	Experimental Diets				
		RS (n = 4)	Vitamin A (n = 4)	RS + Vitamin A (n = 4)	Vitamin E (n = 4)	RS + Vitamin E (n = 4)
Acetate						
Cecal	51.1 ± 20.9	62.2 ± 32.6	45.8 ± 17.4	90.7 ± 13.1*	46.5 ± 8.9	73.0 ± 16.1
Fecal	35.0 ± 11.6	27.0 ± 9.8	30.1 ± 6.5	50.2 ± 19.8	17.3 ± 2.8	31.1 ± 6.1
Propionate						
Cecal	11.5 ± 4.3	11.7 ± 2.6	9.9 ± 2.8	11.5 ± 3.6	8.1 ± 2.5	9.2 ± 2.5
Fecal	9.9 ± 3.6	7.5 ± 4.3	6.4 ± 2.6	2.3 ± 1.2†	5.2 ± 0.9	4.6 ± 3.5
Butyrate						
Cecal	9.4 ± 3.3	13.7 ± 7.2	12.8 ± 4.3	16.6 ± 3.6	7.5 ± 3.3	10.3 ± 4.4
Fecal	10.0 ± 5.0	11.5 ± 5.2	13.0 ± 5.6	14.1 ± 5.6	6.8 ± 2.5	4.6 ± 2.1
Total SCFA						
Cecal	76.8 ± 26.8	89.2 ± 32.9	71.5 ± 17.3	119.7 ± 12.9	65.1 ± 14.3	93.4 ± 19.2
Fecal	60.5 ± 17.0	47.1 ± 11.2	52.2 ± 13.0	67.3 ± 26.0	34.2 ± 5.9	40.8 ± 7.6

a: SCFA concentration is expressed in $\mu\text{mol/g}$ of cecal luminal content or of feces sample. Values are means $\pm$ SD; n, number of rats. SCFA, short-chain fatty acid.

b: Statistical significance is as follows: *, $p < 0.05$; †, $p < 0.01$, by analysis of variance and Tukey's test.

Hepatic Enzyme Activities

NDMA_d activity: NDMA_d activity measured in rats fed experimental diets for four weeks is shown in Table 4. No difference was observed in the different groups.

UDPGT activity: UDPGT activity significantly decreased in rats fed RS and RS + vitamin A diets. The other diets did not modify this activity (Table 4).

Cecal β -Glucuronidase Activity

The results showed that all experimental diets significantly decreased the β -glucuronidase activity (Figure 3); only the vitamin A diet did not affect β -glucuronidase activity.

Fat-Soluble Vitamin Concentrations in Liver and Colon

All the retinyl esters were measured in the liver and colon.

In the liver, only retinyl palmitate, which is the major hepatic form, can be measured as vitamin A. In the diets supplemented with vitamins, the liver concentrations of retinyl palmitate or α -tocopherol were significantly increased (Figure 4, Table 5).

In the colon, vitamin A- and RS + vitamin A-supplemented diets led to the appearance of retinol in colonic epithelial cells, in comparison with the basic diet. No other form of vitamin A was detected in colonic epithelial cells. The results also showed a greater α -tocopherol concentration in colonic epithelial cells from rats fed vitamin E- and RS + vitamin E-supplemented diets than in the animals fed the basic diet (Figure 4, Table 5).

Discussion

The present study was undertaken to determine the modifying effect of RS and the fat-soluble vitamins A and E on hepatic, cecal, and fecal parameters involved in the formation of carcinogens in the colon.

The hepatic enzyme activities measured in this study occur in the xenobiotic metabolism. The NDMA_d activity depends on the CYP450 2E1 isoform. This isoform may be implicated in the hepatic DMH metabolism (37,38). Several authors have reported modifications of the 2E1 isoform level in rats fed carrot fibers (39) and *in vitro* modification of the NDMA_d activity by retinoid (40). In this study, none of the experimental diets modified the NDMA_d activity. After metabolism by phase I-metabolizing enzymes, xenobiotics are conjugated by phase II enzymes. The UDPGT activity occurs in the conjugation of methylazoxymethanol (DMH me-

Table 4. Effects of Experimental Diets on Hepatic Enzyme Activities^{a,b}

	Basic Diet (n = 4)	Experimental Diets				
		RS (n = 4)	Vitamin A (n = 4)	RS + Vitamin A (n = 4)	Vitamin E (n = 4)	RS + Vitamin E (n = 4)
NDMA _d	4.09 ± 1.80	3.34 ± 1.66	5.28 ± 1.34	4.44 ± 2.48	5.30 ± 0.99	4.04 ± 0.75
UDPGT	1.04 ± 0.19	0.62 ± 0.11*	0.65 ± 0.23	0.62 ± 0.23*	0.78 ± 0.11	1.16 ± 0.26

a: Values are means $\pm$ SD; n, number of rats. NDMA_d, nitrosodimethylamine N-demethylase (nmol HCHO/min/nmol cytochrome P-450); UDPGT, uridine diphosphoglucuronyl transferase (nmol p-nitrophenol/min/mg protein).

b: Statistical significance is as follows: *, $p < 0.05$, by analysis of variance and Tukey's test.

Figure 3. Effects of experimental diets on cecal bacterial β -glucuronidase activity. Vertical bars, SD. PNP, *p*-nitrophenol. Analysis of variance and Tukey's test were used for statistical comparison: *, $p < 0.05$; **, $p < 0.01$.

Figure 4. High-performance liquid chromatography profiles of vitamin A content in hepatic and colonic cells of rats supplemented with vitamin A and RS + vitamin A diets. Injection volumes are 5 μ l for liver extract and 20 μ l for colon extract (it was verified that no retinol peak was found in liver with an injection of 20 μ l).

Table 5. Effects of Experimental Diets on Fat-Soluble Vitamin A and E Concentration in Hepatic and Colonic Cells^{a,b}

	Experimental Diets					
	Basic Diet (n = 4)	RS (n = 4)	Vitamin A (n = 4)	RS + Vitamin A (n = 4)	Vitamin E (n = 4)	RS + Vitamin E (n = 4)
Hepatic cells						
α-Tocopherol, μg/g	60.0 ± 9.1	NM	NM	NM	1,428 ± 516 [†]	1,403 ± 615 [†]
Retinyl palmitate, mg/g	0.65 ± 0.28	0.38 ± 0.11	13.78 ± 3.07 [†]	9.50 ± 1.62 [†]	0.57 ± 0.34	0.82 ± 0.05
Colonic cells						
α-Tocopherol, μg/g	12.38 ± 2.83	NM	NM	NM	43.8 ± 15.0*	45.8 ± 10.2*
Retinol, μg/g	ND	ND	3.12 ± 0.74	2.98 ± 0.79	ND	ND

a: Values are means ± SD; n, number of rats. ND, not detectable; NM, not measured.

b: Statistical significance is as follows: *, $p < 0.05$; †, $p < 0.001$, by analysis of variance and Tukey's test.

tabolite) to glucuronic acid (27,37). The conjugated metabolites are then secreted in the bile before reaching the colon. Our results show that the UDPGT activity is weaker in RS and RS + vitamin A groups than in the basic group and could result in a decrease of conjugated methylazoxymethanol excretion in the colon. In the colon, the level of released methylazoxymethanol also depends on the bacterial activity of β-glucuronidase. This enzyme is largely responsible for the hydrolysis of glucuronide conjugate in the gut and thus is important in the release of carcinogenic substances (33). Only diets containing RS and/or vitamin E are responsible for a decrease of the cecal β-glucuronidase activity. The other cecal and fecal parameters observed in this study, such as fecal output and cecal weight and pH, are also modified by RS in the diet. Our results confirm that RS has effects on the colon similar to those of nonstarch polysaccharides, as noted by Phillips and collaborators (21). Moreover, according to Gee and co-workers (41), who used Eureka RS, the RS-supplemented animals in our study had a significantly higher output than the basic group. This result is similar to those of Lupton and Kurtz (26), who used wheat bran: the fecal output of rats fed wheat bran was greater than that of rats fed a fiber-free diet. We observed also an increase of the fermentative activity in the cecum and a fall of the pH between 0.7 and 1 pH unit in the rats supplemented with RS; these results are in accordance with those of Gee and co-workers and Lupton and Kurtz.

In our study, only the vitamin A-supplemented diet significantly reduced the total number of AC, although no modification of the activities of phase I and II xenobiotic-metabolizing enzymes and β-glucuronidase was observed. The preventive effect of vitamin A is probably due to a direct effect of this fat-soluble vitamin on colonic epithelial cells. Indeed, retinol was found in colonic epithelial cells of rats fed vitamin A. *In vitro*, several authors showed that retinoids inhibited the action of numerous mutagenic substances (42,43). We recently noted that *in vitro* retinol has an antimutagenic activity toward methylazoxymethanol in *Salmonella typhimurium* TA100 strain (44).

Previous studies with diets containing RS or butyrate showed variable results. Sakamoto and collaborators (43) observed that RS increased butyrate production in the cecal, distal, and fecal contents. The butyrate concentration was

increased in the cecal contents but not in the fecal contents of rats fed a cornstarch diet for 30 days (46). In the same study, the authors showed that fecal butyrate concentration was increased after 105 days on the cornstarch diet. Although Sakamoto and co-workers observed a greater butyrate concentration in feces of rats fed RS, no inhibitory effect was shown on colon carcinogenesis. Another study showed a protective effect of butyrate against large bowel cancer in experimental colitis (47), and yet another study reported that oral intake of butyrate enhanced the incidence of colon carcinogenesis in rats (48). However, a previous *in vivo* study showed that fiber associated with high butyrate production in the distal colon was protective against colon cancer (49). In our study, after four weeks, no increase of butyrate was observed in cecal and fecal contents of rats fed RS-supplemented diets. This result could account for the finding that the RS-supplemented diet did not significantly decrease the total number of AC per rat. Although we did not observe an increase in SCFA production in the cecum, we showed a decrease in pH of rats fed RS. This surprising result and the weak β-glucuronidase activity, the great fecal output, and the high cecal weight observed in groups fed RS do not seem to be parameters affecting the initiation of AC.

β-Glucuronidase activity was also inhibited in rats fed the vitamin E diet, but this decrease did not result in fewer AC. The presence of α-tocopherol in the colonic epithelial cells did not protect the cells against the action of methylazoxymethanol. Although several authors have shown an *in vitro* antimutagenic activity of α-tocopherol (42), we have observed slight effects against the mutagenic activity of methylazoxymethanol in *Salmonella typhimurium* TA100, only when bacteria were cultured in a medium containing vitamin E (44). α-Tocopherol seems to be unable to protect cells against the action of mutagenic agents, in contrast to retinol.

The association between RS and vitamin A in the same diet leads to about the same protective effect of the vitamin A diet toward AC. The highest level of acetate in cecal contents of rats fed RS + vitamin A did not influence the number of AC formed. Moreover, the fecal output is slightly lower in these groups than in the two other groups containing RS (−22% in comparison to the RS group and −16% in comparison to the

RS + vitamin E group). These differences could result in a better starch fermentation in the cecum, leading to more cecal acetate. It is possible that vitamin A binds to the indigestible starch and is eliminated in the feces. However, we have not measured significant differences between concentrations of vitamin A in colonic and hepatic cells of rats fed the RS + vitamin A diet and the vitamin A diet.

We have measured the same concentration of retinol in the colonic cells for vitamin A and RS + vitamin A diets, so we should have observed the same effect on ACF. However, the vitamin concentration was determined after extraction, and it is possible that retinol in the colonic epithelium of the rats supplemented with the RS + vitamin A diet was less available.

On the contrary, the RS-vitamin E association leads to a decrease of the effect of vitamin E on the AC induction (-36%). No difference of α -tocopherol concentrations in colon and liver between RS + vitamin E and vitamin E groups has been observed. In this model, the beneficial effect of the RS-vitamin E association on the number of AC in comparison with the group fed vitamin E alone could be followed by a very important inhibition of the β -glucuronidase activity.

In this report, it was concluded that vitamin A protects against initiation of colon cancer and that vitamin E has no beneficial effect. RS alone (retrograde high-amylose starch, type 3, used in this study) does not protect against the initiation of colon cancer but seems to be able to attenuate the effect of DMH on colonic mucosa. This lack of significant effect could be due to the fact that butyrate concentration is not increased in the lumen of rats fed RS. On the contrary, in an earlier report we noted that RS and RS + vitamin A produced higher butyrate concentrations and had a more protective effect against the promotion stage of colon cancer than vitamin A alone (23). Feeding a diet containing RS for four weeks is probably insufficient for the colonic microflora to adapt itself to the diet. Further experimental studies are needed to show whether RS inhibits initiation of colon cancer, as well as mechanisms of action. The association between RS and fat-soluble vitamins does not offer cumulative effects.

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