Dietary Inulin Suppresses Azoxymethane-Induced Aberrant Crypt Foci and Colon Tumors at the Promotion Stage in Young Fisher 344 Rats


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ABSTRACT This study was designed to determine the effect of 10% dietary long-chain inulin on the azoxymethane (AOM)-induced colonic preneoplastic aberrant crypt foci (ACF) and small intestinal and colon tumors at the initiation (I), promotion (P) and I + P stages (20 rats per treatment) in Fisher 344 male weanling rats. After an acclimatization period of 1 wk, groups of Fisher 344 male weanling rats were assigned to consume AIN 93G diet (control) or AIN 93G diet containing 10% inulin. All the rats received 16 mg/kg body AOM dissolved in saline subcutaneously at 7 wk of age followed by a second injection at 8 wk of age. An additional group of five rats received only saline and consumed the control diet. The rats received the assigned diets until asphyxiation by CO2 at 16 wk of age for the ACF experiment and 45 wk for the end-point tumor experiment. Feed intake, weight gain, diarrheal index, cecal weight, cecal pH, ACF and tumors in the colon were determined. Rats fed inulin had diarrhea after 2 wk of feeding and recovered by ~4 wk. Cecal weight was greater in rats fed inulin and cecal pH was lower. The inulin group had more than 66% fewer aberrant crypts and 60% fewer ACF compared with the control group. Tumor incidences in the small intestine and colon of rats in the control, I, P and I + P groups were: 78, 31, 0 and 11% and 90, 73, 69 and 50%, respectively. The corresponding values for the distal portion of the colon were 87, 63, 45 and 33%, respectively. Colon tumors per tumor-bearing rat were 4.2, 3.09, 1.36 and 1.2 for the control, I, P and I + P groups, respectively. All groups differed, P < 0.05. The results of this study indicate that dietary long-chain inulin suppresses AOM-induced ACF formation, an early preneoplastic marker of colon tumorigenesis in rats, and colon tumors, particularly at the promotion stage. J. Nutr. 132: 2809–2813, 2002.

KEY WORDS: ● azoxymethane ● aberrant crypt foci ● inulin ● colon tumors ● Fisher 344 rats

Colorectal cancer is the second leading cause of death in the United States (1). It has been suggested that changes in the amount and type of fiber can modify cell proliferation and the etiology of colon cancer, colorectal cancer in particular. Early epidemiological studies suggested that dietary fiber could be protective against colon cancer. The precise mechanism by which fiber exerts its influence is still elusive. Bacterial degradation of fiber in the digestive tract produces butyric acid, which fiber exerts its influence is still elusive. Bacterial degradation of fiber in the digestive tract produces butyric acid, which is absorbed and reduced cancer risk (2). Epidemiological and animal model studies indicate that the etiology of colon cancer is multifactorial and complex (3). Studies in our laboratory (4) and elsewhere (5) have shown that bifidobacterium may be an effective suppressor of colon tumorigenesis, and the growth of bifidobacteria is stimulated by certain “bifidogenic” factors, particularly some oligosaccharides (6,7). This new class of phytochemicals that includes inulin and fructooligosaccharides of various chain lengths is bifidogenic and has been referred to as “prebiotics” (8,9). Hence, prebiotics may alter the large intestinal microbiology and metabolism, resulting in the modulation of colonic disorders including cancer.

Fructooligosaccharides (FOS)3 are composed of glucose-(fructose)n with β-2→1 linkage between the fructose monomeric units, and as polyfructose. The length of the fructose chain varies from 2 to 60 with an average degree of polymerization of > 10. Inulin consists of higher chain-length FOS (10). A number of foods, such as garlic, onion, artichoke and asparagus, have high levels of FOS. Because the β-2→1 glycosidic bond is resistant to hydrolysis by the intestinal enzymes, these oligosaccharides are broken down into their monomers by colonic microflora and are excellent substrates for bifidobacteria (9). Fructooligosaccharides have been shown to be indigestible by enzymes in the human small intestine but are fermented extensively by large-bowel microflora (9,11) to short-chain fatty acids (SCFA), which can be absorbed and metabolized by the host. Bacterial fermentation of chichory fructans and other oligosaccharides produces SCFA in the

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3 Abbreviations used: ACF, aberrant crypt foci; AOM, azoxymethane; FOS, fructose-oligosaccharides; I, initiation; P, promotion; SCFA, short-chain fatty acids.
colon, including a small amount of butyric acid (10,12), which have been shown to increase apoptosis in colon cancer cell lines (13). Furthermore, there are studies demonstrating that cultures of bifidobacteria increase the host’s immune response (14). There is preliminary evidence in experimental animals of a preventive effect of inulin against colon cancer (15–17). Most of the preliminary evidence concerning the influence of inulin has been generated using the rat aberrant crypt foci (ACF) model. There are no data on end-point tumorigenesis or whether the anticancer effect of inulin in the colon is at the initiation and/or the promotion stages.

This study was designed to determine the potential inhibitory influence of long-chain inulin on azoxymethane (AOM)-induced ACF and on the process of tumorigenesis at the initiation (I), promotion (P) and the initiation plus promotion (I + P) stages in the rat colon, and its effect on diarrhea, cecal pH and weight, and fecal bifidobacterial counts. ACF are putative lesions that occur in the colon of both rats and humans. ACF are induced by treatment of rats with colon carcinogens and are considered to be early precursor lesions of colon tumors (18). ACF lesions similar to those found in carcinogen-treated rodents have been observed in colorectal cancer patients (19,20).

MATERIALS AND METHODS

Experimental procedure. After 1 wk of acclimatization, 25 male Fisher 344 (Charles River Laboratory, Wilmington, MA) weanling rats (expt. 1) were divided into two groups and assigned to one of the following two diets until 16 wk of age: AIN-93G (21) (Control-C, 15 rats) and AIN-93G with 10 g/100 g inulin added at the expense of cornstarch (10 rats). Long-chain inulin was obtained from Orafti (Tienen, Belgium) as Raffilin, which was extracted from chicory roots and showed an average degree of polymerization of 40. Temperature and relative humidity were maintained at 21 ± 1°C and 50%, respectively. Light and dark cycles were 12 h each. Rats consumed feed and water ad libitum. Weekly body weights and daily feed intakes were recorded. The diets were prepared fresh every week and stored at 4°C until fed. All the protocols involving rats have been approved by the Institutional Animal Care and Use Committee of Alabama A&M University, Normal, AL.

In expt. 2, 60 rats were divided into three groups of 20 rats each and fed 10 g/100 g inulin during the L, P, and I + P stages of carcinogenesis. The compositions of the experimental diets (AIN-93 based) were the same as in the previous experiment and inulin was obtained from the same source. Ten rats received the control diet (no inulin). In the initiation (I) group, rats received inulin in the diet 3 wk before injection 1 and until 1 wk after injection 2 (5 wk total). Rats were then switched to the control diet. In the promotion (P) group, the rats received the control diet until 10 wk of age (2 wk after the 2nd injection) followed by the inulin for 34 wk. In the I + P group, rats received inulin in the diet through out the 41-wk experiment. The rats were switched to an AIN 93M-based (21) diet at 20 wk of age. The experimental diets and the control diet were fed up to 45 wk of age and then the rats were killed by CO2 asphyxiation. Experimental conditions were the same as in expt. 1.

Diarrheal index. Diarrheal index was measured by assigning the following numbers based on the appearance of the pellets: 0, normal; 1, mild diarrhea; 2, moderate diarrhea (semi solid pellets); 3, overt diarrhea (pasty pellets); and 4, severe diarrhea (watery feces).

Carcinogen injection. All animals received a subcutaneous injection of AOM in saline (Sigma, St. Louis, MO) at 16 mg/kg body at 7 and 8 wk of age according to the standard protocol (22). An additional five rats received an injection of saline alone and consumed the control diet.

Colon sample collection. The colons were removed and flushed with potassium phosphate buffer (0.1 mol/L, pH 7.2). In expt. 1, colons were scored for ACF. In expt. 2, at 45 wk of age, all the rats were killed and colon tumors were characterized (23). Tumor samples were analyzed for number and size.

Cecal weight and cecal pH. The cecum from each rat was excised, weighed and split open, and the pH of the cecal contents was recorded.

Counting the ACF. ACF in the colon were counted as described by Bird (24). Briefly, each colon was split open longitudinally and placed on a filter paper with the luminal surface open and exposed. Another filter paper was placed on top of the luminal surface and fixed overnight using 10% buffered formalin. Each fixed colon was cut into proximal and distal portions of equal length and each portion was further cut into 2-cm long segments. Each segment was placed in a Petri dish and stained using 0.5% methylene blue solution for 5 min. The segments were transferred to another Petri dish containing buffer to remove excess stain, and then examined under a light microscope to score the total number of ACF as well as the number of crypts per focus. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from lamina to basal surface of cells and the easily discernible pycnolipheal zone.

Statistical analysis. Data are expressed as the means ± SEM. Differences were considered significant at P < 0.05. Differences between control and inulin-fed groups and proximal vs. distal colon were tested by Student’s t test and paired t tests, respectively. Other data were analyzed by ANOVA with post-hoc Tukey’s tests. All analyses were performed using the SAS Statistical packages (SAS Institute, Cary, NC) (25).

RESULTS

Experiment 1

Feed intake, body weight and cecal weights. Body weight gains of rats tended to be lower (P < 0.08) in the inulin group than in the control group, although the inulin group tended (P < 0.08) to consume more food. Cecal weights were higher in the inulin group than in the controls (P ≤ 0.001), as expected (Table 1).

Diarrheal index. Inulin induced diarrhea. The first sign of diarrhea in the inulin group appeared immediately after wk 2 of feeding and peaked at wk 3 (age 7 wk) followed by recovery from diarrhea by the end of the wk 4 (age 10 wk). Other than the diarrhea described above, stool consistency was firm (pelted) throughout the study with no visible differences between groups. There were no signs of diarrhea in the control group.

Aberrant crypt foci. The rats administered saline (vehicle), showed no evidence of ACF formation in the colon (data not shown). In the rats fed the control diet, AOM induced an average of ~150 ACF/colon (Fig. 1). Rats fed 10 g/100 g inulin had reductions in ACF in the colon by 62.5% in the proximal and by 60.1% in the distal colon (Fig. 1) with an overall 60.1% reduction in ACF (P < 0.001), compared with

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Inulin</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Weight gain, g/16 wk</td>
<td>228 ± 2.59</td>
<td>210 ± 3.28</td>
</tr>
<tr>
<td>Feed intake, g/d</td>
<td>16.0 ± 0.26</td>
<td>19.0 ± 0.30</td>
</tr>
<tr>
<td>Cecal weight, g</td>
<td>0.84 ± 0.05</td>
<td>3.13 ± 0.05*</td>
</tr>
</tbody>
</table>

* Values are means ± SEM.

* Different from control, P < 0.001.
controls (Fig. 1). Rats fed inulin had 66% fewer total crypts in the colon (data not shown). The distal colon had more ACF and total crypts ($P < 0.01$) than the proximal segment, consistent with reports that the distal colon has a greater colon cancer incidence than the proximal colon in humans (22). The total numbers of foci containing 1, 2, 3, 4 and $\geq 5$ crypts/focus were counted in the proximal and distal regions of the colon (Table 2). The foci with 3, 4 and $\geq 5$ crypts were fewer ($P < 0.001$) in the rats fed inulin than in the control group.

**Experiment 2.** Rats consuming inulin for longer periods (P and I + P stages) gained slightly more weight ($P < 0.05$) than the controls and the rats consuming inulin only at the initiation stage (Table 3). Feed intake did not differ among groups. Cecal weights were greater ($P < 0.01$) in rats consuming inulin for longer periods than in the control rats and rats consuming inulin only at the initiation stage. Interestingly, cecal weights in rats consuming inulin for 5 wk at the initiation stage were much higher ($P < 0.01$) than those in the control group. Cecal pH was inversely related to cecal weight ($R^2 = 0.921$, $P \leq 0.01$) and long-term inulin feeding reduced cecal pH significantly. During the initial weeks of feeding diets containing 10 g/100 g inulin, the rats exhibited diarrhea but adapted quickly as in expt. 1 and had slightly greater weight gains than the controls (Table 3). Individual susceptibility of rats to AOM and high fiber diets may have attributed to the deaths seen in the expt. 2 (Table 4).

**Tumor incidence.** There was a lower ($P < 0.02$) tumor incidence in rats in the P and I and I + P compared with the controls (Table 4). There were no differences in tumor numbers in the I group compared with the control group with 11/34 and 23/34 tumors occurring in both groups in the distal and proximal colon, respectively. The percent incidence (Table 4) of colon tumors in the distal colon in the control group and the groups fed 10% inulin at the I, I + P stages is shown in Table 4. Tumor numbers did not differ ($P < 0.05$) in the P and I + 1 groups in the proximal or distal colon (Table 4); however, there was a difference ($P < 0.02$) between the control and I and P and I + P groups. There were more tumors/tumor-bearing rats in the controls than in all other groups and in the I group than in the P or I + P groups, which did not differ. Tumor size differed among groups in the order C$>1>P>I+P$ (Table 4).

**DISCUSSION**

This study was conducted to evaluate the potential inhibitory properties of inulin against formation of AOM-induced colonic ACF, which are putative preneoplastic lesions. Dietary inulin inhibited AOM-induced colonic ACF formation in Fisher 344 male rats. The precise mechanism by which inulin inhibits preneoplastic lesions of the colon is not fully known. Body weight gain of rats fed the inulin-containing diet was less than in those fed the control diet. Campbell et al. (12) reported an initial decline in weight gain of rats after feeding the experimental diet (FOS), which could have been due to the change from a nonpurified to purified diets. However, they did not report any significant differences in body weights at the end of the study. Other studies (4,5,26) reported no significant influence of dietary nondigestible carbohydrates or inulin on weight gain or feed intake of rats. In previous studies in which rats were fed inulin, no data on diarrhea were provided (5,26) However, in one human study, inulin fed at 15 g for 45 d resulted in increased stool frequency, excretion of wet and dry matter, nitrogen and energy, with increases in wet matter and nitrogen being significant (6). Challa et al. (4) reported a higher diarrheal index in rats fed lactulose and bifidobacteria than in the control groups. In expt. 2, inulin affected both cecal weight and cecal pH. Previous studies by others showed similar effects of FOS (12,26–29). The elevation in cecal weight due to ingestion of inulin or FOS diets may result from SCFA promoting cecal growth. The acidic cecal pH is probably caused by the greater level of SCFA production (12). Rowland et al. (26) demonstrated a significant increase in cecal weight (20–32%) in rats fed 5% inulin and a significant decrease in cecal pH. They suggested that consumption of inulin was associated with potentially beneficial changes in cecal physiology and bacterial metabolic activity in relation to tumor risk and the incidence of putative preneoplastic lesions in the colon. Although fecal pH was not measured in that

**TABLE 2**

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>$\geq 5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>6.3 ± 1.06</td>
<td>22.1 ± 0.96</td>
<td>60.0 ± 0.88</td>
<td>39.5 ± 1.50</td>
<td>26.3 ± 1.40</td>
</tr>
<tr>
<td>Inulin</td>
<td>10</td>
<td>5.0 ± 0.50</td>
<td>16.0 ± 0.69</td>
<td>24.0 ± 1.77*</td>
<td>10.0 ± 0.71*</td>
<td>5.2 ± 0.60*</td>
</tr>
</tbody>
</table>

* Values are given as means $\pm$ SEM.
* Different from control, $P < 0.001$. 

**FIGURE 1** Effect of 10% dietary inulin on colonic aberrant crypt foci (ACF) in Fisher 344 male rats (expt. 1). Values are means $\pm$ SEM, n = 15 (control) or 10 (inulin). #Differentiate between proximal and distal, $P < 0.001$. *Different from control, $P < 0.001$. 

**TABLE 3**

Effect of dietary inulin on number of aberrant crypts/focus in colon of rats fed control and 10 g/100 g inulin diet for 13 wk (expt. 1)"
study, a reduction in cecal pH is indicative of fecal pH reduction. Fecal pH has been suggested to be a possible factor in suppression of colon tumorigenesis (12). The bifidogenic effect of inulin and oligofructose has been shown by many researchers (8–11). Dramatic positive changes in microflora composition have also been shown in human studies (6,27) at inulin doses between 5 and 20 g/d for 15 d. It is possible that the metabolites produced by the bifidobacteria may affect the mixed-function oxidases, ornithine decarboxylase and ras p21 expression (16). Studies from our laboratory (4) and those of Kulkarni and Reddy (28) have shown the inhibitory effect of bifidobacteria on AOM-induced ACE. In humans, bifidobacteria have been regarded as beneficial because they stimulated immune function, particularly against malignant cells (6). Reddy et al. (5) also administered 10% long-chain inulin (Raftilin HP) to rats injected with AOM. They observed a statistically significant 35% reduction in the numbers of ACF (> 2 crypts per focus) in the colon. Gallaher et al. (29) administered 2% FOS to rats during the postinitiation stage of carcinogenesis induced by 1,2-dimethylhydrazine. They observed a significantly reduced the total number of colon tumors induced was reduced significantly in the P and I + P groups but not in the I group alone. However, colon tumors per tumor-bearing rat and colon tumor size were reduced significantly in the I group compared with the control group. The effects of inulin were much more pronounced in the P group. Overall, feeding inulin at the P stage resulted in substantial reduction in colon tumorigenesis induced by AOM. Small intestinal tumors were reduced dramatically by inulin when fed at the P stage.

In conclusion, feeding inulin significantly reduced the total number of ACF in male Fisher 344 rats. The inhibition by inulin may be because of a change in the colonic microecology resulting from the fermentation of the fructan. Because the long-chain oligosaccharides are fermented at a slower rate than short-chain oligosaccharides, they indeed may reach the more distal part of the colon where they can stimulate microbial metabolism. Altering the metabolic activity of the colonic microflora by inulin, which is “bifidogenic,” reduction in cecal pH and stimulation of immune activity may be the mechanisms by which the anticarcinogenic effect is exerted. The results indicate that feeding inulin inhibits ACF formation, an

### TABLE 3

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Weight gain g/100 g</th>
<th>Feed intake g/d</th>
<th>Cecal weight g</th>
<th>Cecal pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>10</td>
<td>344.0 ± 3.14b</td>
<td>17.0 ± 0.32</td>
<td>0.94 ± 0.05c</td>
<td>7.18 ± 0.14a</td>
</tr>
<tr>
<td>I</td>
<td>20</td>
<td>355.4 ± 3.8b</td>
<td>19.2 ± 0.60</td>
<td>2.24 ± 0.15b</td>
<td>6.98 ± 0.32a</td>
</tr>
<tr>
<td>P</td>
<td>20</td>
<td>363.8 ± 4.5a</td>
<td>19.0 ± 0.48</td>
<td>3.08 ± 0.78a</td>
<td>6.00 ± 0.12c</td>
</tr>
<tr>
<td>I + P</td>
<td>20</td>
<td>368.0 ± 4.1a</td>
<td>19.5 ± 0.51</td>
<td>3.12 ± 0.95a</td>
<td>5.90 ± 0.09c</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Means in a column without a common letter differ, P < 0.05.
2 Abbreviations used: C, Control; I, initiation; P, promotion.

### TABLE 4

<table>
<thead>
<tr>
<th>Group</th>
<th>N1/N03</th>
<th>Rats with colon tumors</th>
<th>Proximal</th>
<th>Distal</th>
<th>Proximal</th>
<th>Distal</th>
<th>Total</th>
<th>Tumors/tumor bearing rat</th>
<th>Tumor size Rats with small-intestinal tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>8/9</td>
<td>90</td>
<td>37.5</td>
<td>87</td>
<td>11a</td>
<td>23a</td>
<td>34a</td>
<td>4.25 ± 0.50a</td>
<td>8.1 ± 0.96a</td>
</tr>
<tr>
<td>I</td>
<td>11/15</td>
<td>73</td>
<td>36.3</td>
<td>63</td>
<td>11a</td>
<td>23a</td>
<td>34a</td>
<td>3.09 ± 0.46b</td>
<td>4.3 ± 0.57b</td>
</tr>
<tr>
<td>P</td>
<td>11/16</td>
<td>69</td>
<td>18.1</td>
<td>45</td>
<td>5b</td>
<td>10b</td>
<td>15c</td>
<td>1.36 ± 0.24c</td>
<td>2.3 ± 0.19c</td>
</tr>
<tr>
<td>I + P</td>
<td>9/18</td>
<td>50</td>
<td>11.1</td>
<td>33</td>
<td>4b</td>
<td>7b</td>
<td>11c</td>
<td>1.2 ± 0.17c</td>
<td>1.7 ± 0.05d</td>
</tr>
</tbody>
</table>

Note: (n = 39).
1 Values are means ± SEM, or %. Means in a column without a common letter differ (Tukey’s test), P < 0.05.
2 Abbreviation used: C, Control; I, initiation; P, promotion.
3 N1, rats with tumors; N0, total number of rats at killing.
early preneoplastic marker of malignant potential in the process of colon carcinogenesis. This study provides evidence that dietary inulin suppressed AOM-induced colon tumorigenesis in Fisher 344 male rats, especially at the P stage and, therefore, colon tumorigenesis may be highly sensitive to dietary intervention. Adults who may have preneoplastic lesions in their colon may therefore benefit from dietary long-chain inulin.

LITERATURE CITED