Proliferative Activity in the Colon of the Mouse and Its Modulation by Dietary Starch, Fat, and Cellulose

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ABSTRACT

The effect of dietary starch, fat, and cellulose on colonic proliferation was studied in female C57Bl/6J mice after 4 weeks of feeding with diets containing various levels of starch (3, 36, and 57-65%), various levels of fat (corn oil, 5 and 29%) and various levels of cellulose (2 and 10%). Cell proliferation was measured by colchicine arrest and [3H]thymidine incorporation. The following parameters were analyzed: mitotic index, labeling index, and position of labelled cells along the crypt.

Increasing starch content from 3 to 36% decreased cell proliferation both in low (5%) and high (29%) fat diets. By estimating simultaneously the effects of starch and fat with a single multiple regression model, we observed a decrease of mitotic index from 3.04 ± 0.34 to 2.04 ± 0.43 (means ± SE) (P < 0.05) when starch was increased from 3 to 36% regardless of the level of fat. Other proliferation parameters showed a similar pattern. Changes in dietary fat alone did not affect significantly cell proliferation. We also investigated the effect of starch at high levels (57-65%) and its interactions with cellulose. High starch (57-65%) increased the labeling index from 7.70 ± 0.58 to 9.65 ± 0.88 (P < 0.05), when also considering the effect of cellulose in the multiple regression model. Cellulose by itself did not change the labeling index. Varying starch from 36 to 57-65% increased the number of cells/crypt column from 22.20 ± 0.82 to 25.87 ± 1.21 (P<0.05) and varying cellulose from 2 to 10% increased the number of cells/crypt column from 22.20 ± 0.82 to 27.25 ± 0.97 (P < 0.01).

The results indicate that either high or low fat diets, containing 36% starch, have the minimum proliferative effects in the mouse colon. However, diets containing high levels of both starch (57%) and cellulose (10%) may induce an increase in proliferation. These data suggest a potential beneficial effect of starch on colon proliferation.

INTRODUCTION

Epidemiological data from different populations suggest that diets rich in lipids and relatively poor in fiber and complex carbohydrates may increase the incidence of colon cancer (1-3). Results with experimental animals indicate a relationship between diet and colon cancer (4-8).

To explain the link between dietary variations and colon cancer it has been suggested that some dietary components act as promoters modifying the proliferation of the cells of the intestinal mucosa (9-11). This hypothesis is based on epidemiological and experimental studies which demonstrate an association between increased proliferation and a high incidence of colon cancer (12-15).

Experimental data on mice have shown that proliferative activity in colon mucosa is increased by administration of a bolus of fat (11), and by intrarectal administration of free fatty acids and bile acids (10, 16). Increased mucosal proliferation was also observed after feeding high fat diets to mice for 10-15 weeks (17), while similar diets fed for a shorter period did not change colon proliferation (18, 19). Additional studies have demonstrated that the effects of fiber on colon mucosa proliferation are actually more complicated than these first studies indicated and vary according to the fiber used (7, 20).

Recently we demonstrated in mice that starch, cellulose, and minerals, but not sucrose, casein, and vitamins, counteract the proliferative activity caused by a bolus of fat, while high fat diets low in starch, cellulose, and calcium increased cell proliferation in colonic mucosa (21).

Most previous studies on diet and proliferation had focused on lipids, fibers, and minerals (7, 11, 22) and little is known about starch; it therefore seemed of interest to study the effect of starch, fat, and cellulose on colon proliferation in C57Bl/6J mice.

MATERIALS AND METHODS

Animals. In all the experiments 11-13-week-old female mice (C57Bl/6J, Charles River SpA, Italy) were used. The animals were housed in plastic cages with wire tops and bottoms.

Diets and Treatments. The composition of the diets used in the experiments is given in Tables 1 and 2, with the indication of caloric content and mean caloric intake/mouse/day of the different dietary treatments. Starch was added in the diets with a concomitant reduction of sucrose. For this reason the diets referred to as high, medium, and low starch diets may be also considered, respectively, low, medium, and high sucrose diets. The high fat diets of Table 1 differ in caloric content from the low fat diets by about 25%. In fact, we could not design a scheme of dietary treatments, combining high and low levels of starch and fat, that could be perfectly balanced in caloric content. The diets of Table 2 had a maximum variation of caloric content of about 11%. We also calculated the caloric intake of animals in the different treatment groups by daily weighing the food consumed by individual animals in metabolic cages. The mean caloric intake/mouse/day in animals on different dietary treatments varied from a minimum of 14.06 to a maximum of 18.06 kcal/mouse/day. Changes in caloric intake might theoretically introduce a not-controlled variable in proliferation experiments. In our results, however, caloric intake and labeling index were not correlated (r = 0.23); so, caloric intake was not considered in the analysis of data. In the diets used in the experiments which focused on the effect of starch and cellulose (Table 2), cellulose was added with a concomitant reduction of sucrose in the low and medium starch diets, or with a reduction of starch in the high starch diet. Notwithstanding the high fat content, diets containing 29% fat were soft, but still solid.

For statistical analysis of the data, diets containing 57 or 65% starch were both considered "high starch" diets. Experimental groups were composed of 8-18 animals, due to the repetition of some dietary treatments. Animals were fed ad libitum by leaving food in Petri dishes on the wire bottom of the cage. Diets were administered for 4 weeks. The weights of the mice at the end of the feeding period are reported in Tables 3 and 4. All the mice were in good condition. Although some dietary treatments increased weight gain considerably (e.g., in 36% starch-5% fat diet), there was not a simple correlation between caloric content of the diets and weight gain.

After this period the animals were injected i.p. with [3H]thymidine (1 μCi/g body weight, specific activity 42 Ci/mmol, Amersham, UK) and with 1.5 μg/g body weight of colchicine (Sigma Chemical Co., St. Louis, MO) and sacrificed 2 h later by cervical dislocation. The colon was washed with cold saline, fixed in buffered formalin and processed for histology. Paraffin sections (5 μm in thickness) were dipped in...
The labeling index and mitotic index were defined as the number of labeled cells per crypt, which was divided in five equal parts called "sectors." Sector 1 represents the bottom and sector 5 the top of the crypt. The labeling index analyzed as described for a and b.

Tests of the hypothesis: $\beta_1 = 0$ or $\beta_2 = 0$ are interpreted as testing for the effect of one or the other dietary component over both levels of starch and fat.

Fig. 1. Proliferative activity in the colon of mice fed for 4 weeks with diets containing two levels of starch (3% and 36%) and two levels of fat (5% and 29%). a, mitotic index; bars, mean values ± SE. The number of animals is on the right of the bars. Probability values calculated with one-tailed $t$-statistics are reported over the bars. b, multivariate statistical analysis of the data shown in a. The shaded band represents the mean "basal values" ± SE of mitotic index, predicted from the values in a when starch and fat levels are low (3% and 5%, respectively).

The main results obtained with this approach are outlined in the left panel of Figs. 1, 3, and 4, with the probability values obtained for each comparison. The interpretation of the data with this simple approach was sometimes problematic, because of the difficulty of interpreting variable effects of different levels of each dietary component. We therefore decided to utilize a more complicated statistical approach, in which outcome parameters (mitotic index, labeling index, number of cells/crypt) were fitted to an additive linear model by the method of least squares. The independent variables in this multivariate analysis were dichotomous indicators representing the levels of fat, cellulose, and starch in each mouse's diet. By the proper choice of indicator variables (24), it was possible to test for main effects and interactions between different dietary components. For example, a model for mitotic index testing for the effect of starch at two levels of fat (Fig. 1b) had the form:

$$MI = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3$$

where $MI$ is the mitotic index; $x_1$ is an indicator variable having the value 0 for each mouse fed a low starch diet (3%) and 1 for a medium starch diet (36%); $x_2$ is an indicator variable having the value 0 for a low fat diet (5%) and 1 for a high fat diet (29%). $x_3$ has the value 1 only for those fed medium starch and high fat; and $\beta_0$, $\beta_1$, $\beta_2$, and $\beta_3$ are unknown parameters estimated from the data. The predicted value of $MI$ for a low starch, low fat diet is $\beta_0$, while $\beta_1$ represents the additional increment in $MI$ predicted to derive from a change from 3 to 36% of starch and $\beta_2$ is an analogous "effect" of fat. $\beta_3$ represents the additional increment in effect (if any) due to the interaction of starch and fat.

Tests of the hypothesis: $\beta_1 = 0$ or $\beta_2 = 0$ are interpreted as testing for the effect of one or the other dietary component over both levels of starch and fat.
the other component, and a test of $b_3 = 0$ tests for an interaction of the two dietary components.

In the analysis of the effects of three levels of starch with two levels of cellulose we gave the definition of the $\chi$ and $\beta$ parameters: $
\chi_1 = 0$
for starch 3%; $\chi_1 = 1$ for starch 36% and 57–65%; $\chi_2 = 0$ for cellulose 2%; $\chi_2 = 1$ for cellulose 10%; $\chi_3 = 0$ for starch 3% and 36%; $\chi_3 = 1$ for starch 57–65%. In this analysis $\beta$ represented the calculated value for 3% starch–2% cellulose; $\beta_1$ represented the increment induced by an increase of starch from 3% to 36, 57 or 65%; $\beta_2$ the increment of cellulose from 2% to 10%; $\beta_3$ the increment of starch from 3–36% to 57–65%. The text of statistical significance was performed with the $t$ test ($a = 0.95$) of each individual independent variable. The results of these analyses are summarized in the right panels of Figs. 1, 3, and 4 in the following way: a shaded band, labeled “basal values,” indicates the predicted means ± SE of the parameter on the $y$ axis, when all dietary components are at their lowest levels, according to the additive model used. The effect of a change in a dietary component is shown by a solid point with error bars indicating 1 SE. For example in Fig. 1a, the first point indicates that when starch is increased from 3 to 36% the linear model predicts that the mitotic index will fall from 3.04 ± 0.34 (basal values) to 2.04 ± 0.43 due to the change in starch alone. The asterisk means that this change is statistically significant ($P < 0.05$).

The distribution of labeled cells along the crypt was analyzed by comparing the labeling indices in sectors 2 and 3 of the crypt (insert of Fig. 2) with "$r$" statistics and by fitting a logistic model through the experimental points in the five sectors of the crypt with the following formula:

$$Y = \frac{a}{1 + e^{-bx + c}}$$

Least-squares solutions for parameters $a$, $b$, and $c$ of this model were found by iterative calculations using an algorithm of Marquardt (25). However, the logistic model was constrained in such a way that all the data contributed to the estimation of "pooled values" of parameters $a$ and $c$, while different $b$ parameters were estimated for each diet. Parameter $b$ can be considered an exponential slope parameter, and therefore the model forced differences among diets to be expressed as differences in slope. For example, the coefficient of determination ($R^2$) for the full model including the four different diets shown in Fig. 2 was 0.78. Thus the test of the differences between two $b$ parameters ($t$ test with $a = 0.95$) can be interpreted as a test for comparing one diet to another.

**RESULTS**

**Experiments on the Effect of Starch and Fat on Colonic Proliferation.** The effect of starch and fat on colonic mucosa was studied with diets containing two levels of corn oil (5 and 29% w/w) defined as low and high fat diets in Table 1, and containing two levels of starch (3 and 36% w/w).

The experimental results on proliferation are shown in Fig. 1. Mitotic index was reduced both in low and high fat diets by increasing the level of starch from 3 to 36% (Fig. 1a). This reduction was significant with ordinary "$t$" statistics in the high fat group. A similar decrease was observed by measuring the labeling index (Fig. 1c) and was significant in the low fat diets. Using multivariate analysis, and considering simultaneously both levels of starch and of fat (Fig. 1, b and d) the effect of an increase of starch from 3 to 36% was significant ($P < 0.05$) both for mitotic and labeling indices. An increase of fat from 5 to 29% caused an increase of the indices of proliferation, but this increase was not significant either with "$t$" statistics or with multivariate analysis. There was no significant statistical interaction between starch and fat.

In a further analysis we studied the distribution of labeled cells along the crypt in the different diets (Fig. 2). The symbols of the lower part of the figure represent the observed values of the labeling index in different sectors of the crypt for each diet. Labeling index data were fitted to a logistic function (see “Materials and Methods”). This analysis indicated that diets containing 36% starch (as compared to those containing 3%) had lower labeling indices, and those variations were significant ($P = 0.001$); fat content did not affect significantly the distribution of labeled cells along the crypt ($P = 0.11$) (the different values of the slope parameter $b$ are reported in the legend of Fig. 2).

We also analyzed separately the labeling indices in sectors 2 and 3, and the results are shown in the insert of Fig. 2. An increase of starch from 3 to 36% caused a reduction of the labeling index in sector 2 both in low and high fat diets. A similar effect was observed in sector 3, but was significant only for the low fat diet.

**Experiments on the Effect of Starch and Cellulose on Colonic Proliferation.** Given the inhibiting effect of 36% starch on colonic proliferation we continued to study this phenomenon in the low fat diets by further increasing the amount of starch to 57–65% (w/w); an increase in starch was not possible in high fat diets without extensively modifying the other components. We also wanted to study the starch effect on mucosal cells in combination with cellulose.

For this purpose, we fed mice for 4 weeks with diets containing different levels of starch (3, 36, and 57–65%, defined respectively, as low, medium, and high starch diets) and two levels of cellulose (2 and 10%) (see Table 2). Two dietary treatments are the same as in the previous experiment (5% corn oil–3% starch and 5% corn oil and 36% starch). The relevant data obtained are summarized in Fig. 3, a and c. Varying starch from 36 to 65% caused an increase of the mitotic and labeling indices at both levels of cellulose which was significant with

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**Table 2**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Starch (%)</th>
<th>Cellulose (%)</th>
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<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>2</td>
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<tr>
<td>B</td>
<td>5</td>
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<td>C</td>
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<td>10</td>
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<td>E</td>
<td>57-65</td>
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<td>F</td>
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**Fig. 2.** Distribution of labeled cells in five different sectors of the crypt of mice fed 5% fat-3% starch (C), 5% fat-36% starch (B), 29% fat-3% starch (A), 29% fat-36% starch (x). Observed and predicted values for different diets are shown. Predicted values are calculated from a logistic model:

$$Y = \frac{a}{1 + e^{-bx + c}}$$

in which a single parameter, the "exponential slope" $b$ was allowed to vary among diets (see text). The values of $b$ for the different diets were the following (standard errors in parentheses): 5% fat-3% starch = −2.636 (0.393); 5% fat-36% starch = −3.315 (0.504); 29% fat-3% starch = −2.489 (0.364); 29% fat-36% starch = −2.851 (0.439). Insert, the means ± SE of labeling indices in sectors 2 and 3 of the crypts, in different diets. Values of probability calculated with one-tailed $t$ test are reported over the bars.
by raising starch from 36 to 57-65%, this effect being significant only for the labeling index; (d) cellulose and fat did not vary significantly cell proliferation; (e) crypt height was increased in high starch (57-65%) and high cellulose (10% diets).

The outlined results demonstrate an interesting effect of starch on colon proliferation. It should be noted that in our experiments the increase of starch was obtained with a concomitant reduction of sucrose. It might be possible, therefore, that variations in sucrose itself might have some effect on intestinal proliferation. A published report recently showed that starch was able to counteract the burst of proliferative activity induced in the colon by a bolus of fat in mice (21). In the same experiment sucrose did not have any effect on the fat-induced increase of proliferative activity. Moreover, while sucrose is totally absorbed in the small intestine, it has been reported that a portion of starch enter the colon (26, 27). On the basis of these previous studies we are inclined to explain the effects on colon proliferation observed in the present paper with the increase of starch in the diet.

Diet rich in fat and poor in calcium and complex carbohydrates have been associated with increased proliferative activity in the colon of mice (21). The present results show that an increase in starch from a low level (3%) to an intermediate level (36%) reduced colon proliferation, not only in high fat diets (29%), but also when dietary fat was low (5%).

A variation of the distribution of labeling index along the crypt was also associated with an increase in starch from 3 to 36%: mice on 36% starch diets, in fact, had lower proliferation than those on 3% starch in the entire crypt, but particularly in sectors 2 and 3. Variation of the distribution of labeled cells in the crypt have been observed both in experimental studies and in humans (12, 28). These studies demonstrated a shift of proliferation from the lower to the upper sections of the crypts induced by treatment with colon carcinogens, in familial polyposis and in patients with colon cancer. Given these results, it is of interest that in our experiments a normal dietary constituent, like starch, could induce a similar variation has been suggested to be associated with colon carcinoma risk in humans and in mice (12, 22, 28). It should be noted that the labeling index distribution in the crypt described in this paper is in accordance with the labeling patterns in descending and transverse colon of mice reported in the literature (29).

It is also interesting that we could reverse the effects of starch on proliferation, although not for all proliferation parameters, by further raising starch to 57-65%. We were able, therefore,
to identify an optimal concentration of starch in the diet with the potential of minimizing colon proliferation; this optimal concentration seemed to be around 40% in our experiments on C57Bl/6J mice.

A small increase in proliferation, which did not attain statistical significance, was observed by raising the fat content of the diet from 5 to 29% for 4 weeks. Accordingly, other authors did not observe an increased proliferation when feeding high fat diets to mice for 2 weeks (19) or to rats for 4 weeks (18). On the contrary, 10–15 weeks of feeding high fat diets were reported to increase proliferation in mice (17).

The overall effects of high cellulose on proliferation were inconsistent in our experiments, since we observed a reduction of proliferation when starch was 3% and an increase when starch was 36 and 57–65%. On the other hand, the literature data on fibers and colon proliferation also vary due to the use of different fibers and experimental models. Cellulose prevents mucosal atrophy in the colon of mice on fiber-free diets (20) and increases slightly DNA synthesis in the proximal colon of dimethylhydrazine-exposed rats (7). Wheat bran and other more fermentable fibers, like pectin and guar, increase cell proliferation in control rat colon and in animals treated with dimethylhydrazine (7, 30). Our results do not seem to indicate a clear effect of cellulose on colon proliferation of normal C57Bl/6J mice. High cellulose and high starch, on the other hand, clearly increased crypt height in our experiments. The relevance of this phenomenon for gut physiology and its correlation with carcinogenesis are not clear, although an increased crypt height has been considered to be a factor of risk for colon cancer in animals, although not in humans (31, 32).

Several mechanisms may be responsible for the decreased cell proliferation induced by optimal concentrations of starch in our experiments. Starch may vary intestinal pH, the content of total and soluble bile acids, the intestinal transit time, the composition of gut flora, or act through some unknown mechanism. We have no information at present about the possible mechanisms connecting starch in the diet and proliferation, but we believe that diet-induced reduction of colon proliferation might be important. In fact, a high proliferation rate in colonic mucosa may increase the sensitivity of the colon to carcinogens either by affecting initiation events or acting as a promoting factor (13, 14, 33). The effect of starch, if confirmed in humans, might be of interest, since epidemiological studies indicate that starch is a protective factor capable of decreasing the risk of colon cancer (1–3).

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