The Role of Fat and Calcium in the Production of Foci of Aberrant Crypts in the Colon of Rats Fed 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine

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The modulation by dietary fat levels of intestine carcinogenesis is well documented. New developments suggest that calcium ions may also play a role. A rapid bioassay, the induction of foci of aberrant crypts in the colon, was used to explore the interaction between dietary fat and calcium. Male F344 rats 6 weeks of age were placed on diets containing 5 or 20% corn oil, and 0.04 or 0.32% calcium ion, as calcium lactate. Each dietary group was fed 400 ppm 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and negative controls received the diets alone. A positive control group was given 2 mg *N*-nitrosomethylurea (NMU) intrarectally four times in a 2-week period. All rats were killed after 9 weeks. The intestinal tract was rinsed with Krebs-Ringer buffer. After staining a 6-cm segment of the descending colon and rectum with 0.2% methylene blue, foci of aberrant crypts were evaluated microscopically. With PhIP as a carcinogen, the rats on a high-fat, low-calcium level had more foci of aberrant crypts than animals on a low-fat level. With the higher calcium level, there were fewer foci and aberrant crypts, but the effect of fat was still significant. With NMU and a low-calcium level, the effect of fat level was evident. However, with the higher calcium intake, there were considerably more foci of aberrant crypts than on the low-calcium level, and the effect of the dietary fat level was not obvious. Thus, with the dietary carcinogen PhIP, an enhancing effect of high fat intake and a protective effect of a higher calcium intake on the induction of intestinal foci of aberrant crypts could be visualized in a 9-week test. With limited intrarectal administration of NMU, the association with dietary fat was evident on a low-calcium intake. On a high-calcium intake, there was a considerably higher number of aberrant crypt foci, perhaps because of the more advanced carcinogenic process with NMU. —Environ Health Perspect 102(Suppl 6):53–55 (1994)

Key words: food carcinogens, heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), nutrition and cancer, colon cancer, fat and colon cancer, calcium and colon cander, rapid bioassay

Introduction

In the Western world, intestinal cancer is a major disease in men and women. The descending and sigmoid colon is the main part of the intestinal tract affected. International epidemiologic studies have demonstrated a strong modulating effect of the type and amount of dietary fat (*1*). A protective effect was observed as a function of consumption of cereal bran fibers and other types of fiber controlling stool bulk. They therefore have a diluting effect on the amount of carcinogens and promoting agents like bile acids in the colon. Vegetables and fruits are uniformly protective (*2*). There have been suggestions that foods containing calcium might be inhibiting, based on the seminal findings of Lipkin and Newmark (*3*) and Bruce and Wargovich (*4*) that calcium may affect the rate of cell duplication in the intestine, reduce carcinogenesis, and thus account for a protective action.

The genotoxic carcinogens involved in causing the nutritionally linked cancers such as in the breast, colon, and pancreas are not yet fully documented. However, based on the pioneering discovery of Sugimura and colleagues (*5*), that meat frying generated potent mutagens identified as heterocyclic aromatic amines, we have suggested these chemicals might be the genotoxic carcinogens associated with these diseases (*6*). Felton's group (*7*) discovered that among the heterocyclic aromatic amines formed during cooking, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) was present in appreciable amounts. A bioassay demonstrated that PhIP caused mammary gland cancer in female rats and colon cancer, with emphasis on the distal colon, in male and female rats (*8*). Another heterocyclic amine, 2-amino-3-methylimidazo-[4,5-*f*]quinoline, induced a lower yield of colon cancer compared to PhIP but has been a powerful carcinogen of the mammary gland in female rats, and also led to pancreatic neoplasms, among other cancers (*9*).

Bruce and colleagues (*10*) developed special techniques to readily visualize foci of aberrant crypts formed during intestinal carcinogenesis. Aberrant crypts were an early marker, detectable in less than 10 weeks and in some instances less than 4 weeks.

This article deals with an evaluation of the role of amount of dietary fat and cal-

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Table 1. Composition of modified AIN-76A diets with different fat and calcium (Ca) levels.[a]

Ingredient	Low fat, low Ca	High fat, low Ca	Low fat, high Ca	High fat, high Ca
Casein, vitamin free	200	235	200	235
Cornstarch	516.9	325.9	495.3	304.3
Dextrose	130	83	130	83
Cellulose	50	59	50	59
Corn oil	50	235.2	50.	235.2
Calcium lactate $5H_2O$	3.1	3.1	24.7	24.7
Salt mix #200150	35	41.1	35.	41.1
Vitamin mix #300050	10	11.8	10	11.8
Choline bitartrate	2	2.4	2	2.4
DL-methionine	3	3.5	3	3.5

[a]Diets were mixed by Dyets, Inc. (Bethlehem, PA) and shipped and stored at 4°C. Data in the table are g ingredient/kg diet.

cium studied in four different diets on the induction by PhIP of aberrant crypts in male F344 rats. As a control to the effect of PhIP, the intrarectal administration of *N*-nitrosomethylurea (NMU), a reliable procedure to induce cancer in the distal colon, was used.

Materials and Methods

Chemicals

PhIP, with the correct chemical analysis, and purity of 99.9% by HPLC, was procured from Nard Institute (Amagasaki, Japan). NMU was purchased from Ash-Stevens Inc. (Detroit, MI).

Diets

Modified AIN-76A diets were prepared by Dyets, Inc. (Bethlehem, PA). They varied as to the amount of fat as corn oil, 5 and 23.5%, and of calcium ion, 0.04 and 0.32%, levels previously used by Wargovich et al. (*4*). The calcium ion was provided as calcium lactate. The composition of the diets is listed in Table 1.

Treatment of Animals

Male weanling F344 rats were procured from Charles River (Kingston, NY). After being housed in the quarantine unit of The Valhalla Institute, they were culled and randomized in groups of five into 12 experimental and control groups (Table 2). Beginning at 5 weeks of age they were fed one of the four diets. At 6 weeks of age, five rats each in the four dietary groups received their respective diets with 400 ppm PhIP. Another set of five rats each in the four dietary groups were given a solution of 2.0 mg of NMU in 0.35 ml isotonic saline solution adjusted to pH 5.5, by intrarectal infusion up to the splenic flexure utilizing a ball-tipped gastric tube. The NMU infusion was carried out Monday and Thursday or Tuesday and Friday over a 2-week period for a total of 8.0 mg NMU per rat. Untreated control groups of five rats each were fed the respective diets.

The animals were maintained according to our institutional animal care guidelines, the Animal Welfare Act, and the DHHS Guide for the Care and Use of Laboratory Animals. The rats were weighed at the beginning of the test, every 2 weeks thereafter, and at the end. The rats were killed by a CO_2 atmosphere. They were immediately necropsied; the distal colon and rectum were removed and cleansed of fecal material with a 0.2% Krebs-Ringer buffer solution adjusted to pH 7.4. The intestinal tract was cut longitudinally, fixed between two filter papers, and soaked in 10% formalin solution in the buffer. Six-centimeter long segments of the distal colon and rectum were placed on microscope slides and stained with 0.2% methylene blue solution in the buffer for 30 min. The stained tissue was rinsed with buffer and immediately examined by light microscopy at a magnification of 40 or 200 × for foci of aberrant crypts in three segments of 2 cm each. The total number of foci per 6 cm of colon and the total number of aberrant crypts were recorded. The means ± SE were tabulated according to Mantel (*11*) (Table 2).

Results

Administration of 400-ppm PhIP in the diet induced foci of aberrant crypts in the 9-week experimental period (Table 2). Under the conditions of the experiment, almost all rats displayed such foci. On a low-calcium and a high-calcium diet, there were more foci present with a 20% fat diet than with a 5% fat diet. Also, animals on the higher calcium diet had fewer foci and fewer aberrant crypts than animals on the lower calcium diet, irrespective of the dietary fat level. Control animals without carcinogen displayed few or no foci of aberrant crypts.

Rats that were given the carcinogen NMU intrarectally, under conditions where colon cancer was induced at about 30 weeks, displayed a considerable number of foci of aberrant crypts in the 9-week period. On a low-calcium diet, there was also an effect of dietary fat, with more foci and aberrant crypts present in animals on the 20% fat diet compared to the 5% fat diet. However, with the higher dietary calcium level, there was a much larger number of foci and of aberrant crypts. There was no effect of dietary fat under those conditions.

Discussion

This study extends the pioneering findings by Bruce and Bird (*10*) of the value of determining the induction of foci of aber-

Table 2. Foci of aberrant crypts in male F344 rats after 9 weeks on PhIP or NMU.

Group number, diet	Number of rats with foci	Total foci	Total aberrant crypts
1. 5% fat + 0.04% Ca^{2+}, + 400 ppm PhIP	5/5	17 ± 0.6	23 ± 0.6
2. 23.5% fat + 0.04% Ca^{2+}, + 400 ppm PhIP	4/5	30 ± 2.8	55 ± 7.6
3. 5% fat + 0.32% Ca^{2+}, + 400 ppm PhIP	4/5	13 ± 1.6	16 ± 1.8
4. 23.5% fat + 0.32% Ca^{2+}, + 400 ppm PhIP	5/5	20 ± 0.8	34 ± 1.8
5. 5% fat + 0.04% Ca^{2+}	0/5	0	0
6. 23.5% fat + 0.04% Ca^{2+}	1/5	1 ± 0.2	7 ± 1.4
7. 5% fat + 0.32% Ca^{2+}	1/5	3 ± 0.6	5 ± 1.0
8. 23.5% fat + 0.32% Ca^{2+}	0/5	0	0
9. 5% fat + 0.04% Ca^{2+}, +NMU i.r.	5/5	129 ± 6.6	337 ± 21.6
10. 23.5% fat + 0.04% Ca^{2+}, +NMU i.r.	5/5	234 ± 7.0	659 ± 25.4
11. 5% fat + 0.32% Ca^{2+}, +NMU i.r.	5/5	413 ± 6.4	1,123 ± 24.0
12. 23.5% fat + 0.32% Ca^{2+}, + NMU i.r.	5/5	387 ± 7.0	1,173 ± 27.6

Groups of five 6-week-old male F344 rats were given a diet containing 5 and 23.5% corn oil and 0.04% Ca^{2+} as calcium lactate $5H_2O$, in a modified AIN-76A diet. The diet of groups 1 to 4 contained 400 ppm PhIP. Rats in groups 9 to 12 were given intrarectally an isotonic saline solution at pH 6.0 of 2.0 mg NMU twice a week, Monday and Thursday in the first 2 weeks (four doses). After 9 weeks, the rats were killed in a CO_2 atmosphere, their descending colon and rectum was removed, and a 6-cm segment from the anus was analyzed for foci of aberrant crypts as described in "Materials and Methods." Data expressed as mean ± SE, calculated according to Mantel's method (*11*).

rant crypts in the colon of animals given an appropriate carcinogen. An association between aberrant crypts with colon carcinogenesis was also observed by Bruce. A major food carcinogen, PhIP has been studied by Sugimura and Ito (*8,12*), who also observed that virtually all treated animals displayed foci and found about the same number of aberrant crypts per focus as was observed in the present study. However, in the 9-week experimental period, more total foci were found in the present work. Of great interest is the fact that the number of foci and of aberrant crypts was subject to control, in a direct proportion, by dietary fat and in an inverse relationship by dietary calcium. More recently, Alabaster et al. (*13*) noted an association between high- and low-risk diets (high-fat, low-fiber and low-fat, high-fiber, respectively) and eventual colon tumor formation with the colon carcinogen azoxymethane. We found that with a carcinogen of the heterocyclic aromatic amine type present in fried foods, widely prevalent in the human environment, there is an association between dietary conditions favoring or inhibiting carcinogenesis in an abbreviated test determining foci of aberrant crypts.

Interestingly, the widely used direct-acting experimental carcinogen NMU delivered to the descending colon mucosa by intrarectal infusion has been used in studies on the role of diet in this model of intestinal cancer. In the current experiment, a larger number of foci of aberrant crypts, compared to that induced by PhIP, was observed in the 9-week experimental period. In this model, the effect of fat was displayed only with a low-calcium level. With the higher calcium level, such an effect was not found, and a high number of aberrant crypts was observed. It may be surmised that these results might be the consequence of the overall process of carcinogenesis having gone too far in the 9-week period with this carcinogen regimen, so that cells may have been present that are no longer responsive to dietary modulation. In a parallel study where the experimental period was extended to 14 weeks, there was no correlation at all in the NMU model between fat and calcium and the foci with aberrant crypts present (data not shown). At that time, on the high-fat, low-calcium diet, a number of small adenocarcinomas or adenomas of the colon were diagnosed. It will be useful to explore in more detail whether advanced neoplastic cells are less subject to control by dietary fat and calcium. This is potentially of importance in the management of clinical cancer in humans, in which tests are being formulated currently on the control of cancers linked to nutrition, such as cancer of the breast or colon with lower risk diets.

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