

Dietary polyamines promote the growth of azoxymethane-induced aberrant crypt foci in rat colon

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We have examined whether dietary polyamines influence the formation and initial growth of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in rat colon. Effects of a combination of dietary polyamines at three dose levels (putrescine: 50, 280, 740 nmol/g; spermidine: 10, 261, 763 nmol/g; spermine: 1, 31, 91 nmol/g) in the polyamine-poor AIN-76A diet were studied in animals in two different experimental situations: animals treated with AOM alone and animals treated with AOM + difluoromethylornithine (DFMO), a specific inhibitor of endogenous polyamine synthesis. In both experimental situations, dietary polyamines enhanced the growth of ACF, expressed as the number of large ACF (foci with three or more aberrant crypts, ACF ≥ 3), whereas the formation of ACF, expressed as the number of ACF, was apparently not altered. In animals treated with AOM alone, maximal growth enhancing effect on ACF was nearly obtained with the median level of dietary polyamine. In rats fed a low polyamine diet, basic AIN-76A, DFMO reduced the growth of AOM-induced ACF by 83%. This inhibitory effect of DFMO was counteracted by dietary polyamines in a dose-dependent manner, and it was abolished at the highest level of polyamines. In conclusion, it was demonstrated that dietary polyamines are able to enhance the growth of AOM-induced ACF. Further, dietary polyamines reversed the DFMO-caused inhibition of ACF growth, probably by compensating for the DFMO-reduced endogenous polyamine synthesis.

Introduction

Quantification of formation and growth of aberrant crypt foci (ACF*) in the rodent colon has been used as a short-term bioassay to evaluate the role of nutritional components at a very early stage of colon carcinogenesis (1). Several observations have confirmed the putative association between ACF and colon cancer: colon carcinogens induce ACF in rodents (2); ACF are found in resected colonic mucosa of humans at high risk of colon cancer (3); colon cancer chemopreventive agents inhibit the induction and growth of ACF (4); some

***Abbreviations:** ACF, aberrant crypt foci; AC, aberrant crypts; ACF ≥ 3 , ACF with three or more crypts per focus; AOM, azoxymethane; DFMO, D,L- α -difluoromethylornithine; ODC, ornithine decarboxylase EC 1.1.4.17; 1 PA, one unit of dietary polyamines, equal to the polyamine content in the standard B&K rat chow: 230 nmol/g of putrescine, 251 nmol/g of spermidine and 30 nmol/g of spermine; PBS, phosphate-buffered saline.

ACF are dysplastic and putative preneoplastic lesions (5); genetic alterations characteristic of colon cancer are observed in ACF (6,7); and particularly the number of large and fast-growing ACF appears to be associated with the tumor development (8,9). Although ACF are induced by colon carcinogens and share some features of colonic tumors, the association between ACF and colon cancer is not a simple one as the total number and location of ACF do not appear to correlate with the number and location of tumors (10,11).

The polyamines putrescine, spermidine and spermine are present in all cells. Although their exact physiological function remains obscure, several decades of intensive research has revealed involvement of polyamines in cell structure stabilization and in many steps of DNA, RNA and protein synthesis (12). Since polyamines are essential for cell proliferation, interference with the cellular metabolism of polyamines, including exogenous polyamines, is a potential target in cancer chemoprevention and chemotherapy.

Exogenous polyamines from the diet and the intestinal bacteria, which are taken up by the intestine, stimulate cell proliferation and growth (13,14). Several food ingredients contain large quantities of polyamines (14). Difluoromethylornithine (DFMO) is an irreversible inhibitor of ornithine decarboxylase (ODC EC 1.1.4.17), the rate-limiting enzyme in endogenous polyamine biosynthesis. Exogenous polyamines from the diet, intestinal bacteria and the pool of acetylated forms reverse the growth inhibiting effect of DFMO on transplanted tumors (15–20). This is in accordance with the observation that depletion of cellular polyamines elicits compensatory uptake (21) of extracellular polyamines. DFMO is a potential chemopreventive agent in experimental colon carcinogenesis in the rat (22,23). However, the specific influence of exogenous dietary polyamines on tumorigenesis in the colon and on the chemoprevention of DFMO has not been clarified.

In a preliminary study we compared the polyamine content in two standard rat chows and found that the chow yielding the highest number and largest size of carcinogen-induced ACF (2) also had the highest content of polyamines. In the present study we added a combination of polyamines to the polyamine-poor and semisynthetic AIN-76A diet to examine whether: (i) dietary polyamines *per se* promote the formation and growth of AOM-induced ACF in rats; and (ii) dietary polyamines reverse the DFMO-induced inhibition of AOM-induced ACF development by compensating the inhibited endogenous polyamine synthesis.

Materials and methods

Animals and chemicals

Fifty-four male F344 rats obtained from Halan Ltd., UK, weighing 103–129 g at the start of the experiment, were randomized, housed in plastic cages, three animals/cage, in a room with 12 h light–dark cycles, 60% humidity and temperature between 21 and 22°C. Water and diet were given *ad libitum*. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, hexanediamine, and azoxymethane were all purchased from Sigma

Table I. Specific growth rate

Group	Treatment	Specific growth rate
1	AOM + 0 PA	1.01 ± 0.05 ^{a,b}
2	AOM + 1 PA	1.15 ± 0.07 ^{a,f}
3	AOM + 3 PA	1.05 ± 0.05
4	AOM + DFMO + 0 PA	0.74 ± 0.11 ^{b,c,d}
5	AOM + DFMO + 1 PA	0.90 ± 0.07 ^{c,f}
6	AOM + DFMO + 3 PA	0.96 ± 0.11 ^d
7	Vehicle + 3 PA	1.08 ± 0.07
8	Vehicle + DFMO + 0 PA	0.76 ± 0.19 ^e
9	Vehicle + 0 PA	1.07 ± 0.04 ^e

Mean ± SD. $n = 6$.

Means with the same superscript are significantly different ($P < 0.05$).

PA, units of polyamines, see Materials and methods for definition.

$$\text{Specific growth rate} = \frac{\ln w_2 - \ln w_1}{\text{days}} \times 100;$$

w_1 = body wt (g) at the first AOM injection; w_2 = body wt (g) at killing;
days = number of days between recordings of w_1 and w_2 .

Chem. Co. (St Louis, MO), DFMO was a gift from Merrell Dow Research Institute, Strasbourg, France.

Combination of polyamines added to the diet

We mixed a combination of putrescine, spermidine and spermine into the semisynthetic and polyamine-poor AIN-76A diet (Special Diets Services Ltd, Essex, UK). The total and relative amounts of polyamines added to the diet were defined in accordance with a unit system where one unit of polyamines (1 PA) corresponds to the combination of 230 nmol/g of putrescine, 251 nmol/g of spermidine and 30 nmol/g spermine. 1 PA is identical to the levels of these polyamine as determined by us in a standard rat and mouse chow from B&K Universal Ltd, North Humberside, UK. Polyamine analysis of the AIN-76A basic diet (0 PA) showed 50, 10 and 1 nmol/g of putrescine, spermidine and spermine, respectively. Analysis showed that the homogeneity of polyamines in 10 g specimens of the diet varied within 10%.

Experimental design

The influence of dietary polyamines on the formation and growth of AOM-induced ACF in rat colon was examined in two experimental situations: animals treated with AOM alone and animals treated with AOM + DFMO. The concentration of DFMO mixed into the AIN-76A diet was 0.4%, which is 80% of maximum tolerated dose (24). The 54 randomized rats were divided into nine experimental groups of six each (see Table I). All animals were fed AIN-76A diet without any supplements for 1 week followed by the experimental diets that were fed throughout the experimental period. AOM (7.5 mg/kg body weight in 0.9% NaCl), or 0.9% NaCl (vehicle) was given s.c. twice with an interval of 1 week. The first AOM injection was given on day 4 of the experimental diets. The rats were killed 6 weeks after the last AOM injection.

Scoring of aberrant crypt foci (ACF)

Scoring of ACF was carried out as previously described (2). Briefly, the animals were killed by decapitation, the colon removed, rinsed in ice-cold PBS, slit open longitudinally and fixed flat between filter papers for 48 h in a 10% neutral buffered formalin solution, followed by 30-s staining with 0.2% methylene blue dissolved in the same formalin solution. No less than 24 h after staining, the colons were examined in an inverse light microscope. Scoring was carried out blindly. It is assumed that single aberrant crypts form initially, then divide into multi crypt foci. The formation of ACF was expressed as the number of ACF. The growth (multiplication of aberrant crypts within a focus) of ACF was expressed as the number of foci with three or more aberrant crypts, ACF_{≥3}.

Calculation of specific growth rates

Body weights were recorded weekly. The individual growth rate was calculated as follows:

$$\text{Specific growth rate} = \frac{\ln w_2 - \ln w_1}{\text{days}} \times 100$$

w_1 = body weight (g) at the first AOM injection; w_2 = body weight (g) at sacrifice; days = number of days between recordings of w_1 and w_2 .

Estimation of polyamines

For estimation of the polyamines in colonic chyme, the colon was emptied by flushing with 10 ml of ice-cold PBS. The colon content was collected,

weighed and homogenized in 4 vol. of 5% trichloroacetic acid (TCA). Hexanediamine was added as an internal standard and the homogenate was kept on ice for 1 h, followed by centrifugation at 2°C for 10 min at 5000 g. The supernatant was stored at -20°C until analyzed. After dansylation, polyamines (25,26) were separated by HPLC, essentially as described by Seiler and Knödgen (27), and quantified by fluorescence detection using a Millennium data program (Waters, Milford, MA).

Ornithine decarboxylase (ODC) assay

The first 20 cm of the small intestine was removed, placed on an ice-cold block, slit open longitudinally and rinsed with ice-cold PBS. The mucosa layer was removed by scraping with a glass slide, homogenized and used for the enzyme assay. ODC activity was assayed by measuring the amount of ¹⁴CO₂ produced from [¹⁴C]ornithine. The assay was otherwise, with small modifications, carried out as described (28,29).

Statistical methods

Since the values of some of the groups were not normally distributed, the Mann-Whitney rank sum test was used to calculate the statistical difference between experimental groups. Spearman rank order correlation test was used to calculate dose-response correlations between the number of units of polyamines added to the diet and the parameters for body growth, formation of ACF and growth of ACF.

Results

ODC activity

In order to verify the effect of the DFMO treatment, the ODC activity of the first 20 cm of the small intestine mucosa, known to have a high basal activity, was estimated. The small intestine was used for ODC analysis since the entire colon was used for the scoring of ACF. Mucosal ODC activity was undetectable in all the DFMO-treated animals, with the exception of one rat (data not shown).

Specific growth rates of the rats

The body growth curves based on weekly records (data not shown) corresponded well with the specific growth rates (Table I) since no weight fluctuations were seen. AOM-treatment alone did not affect the body growth rate. DFMO given in the diet, reduced the body growth rate by ~30% in both untreated ($P = 0.004$) and AOM-treated animals ($P = 0.002$). In animals treated with AOM alone, the growth rate was increased significantly ($P = 0.009$) with 1 unit of dietary polyamines (1 PA), but not with 3 PA. In saline treated animals, 1 PA was not tested, but 3 PA did, as in the AOM-treated rats, not influence the body growth rate significantly. In animals treated with AOM + DFMO, it was a significant positive correlation between the polyamine content of the diet and the specific growth rates of the rats ($r = 0.75$, $P = 0.002$).

The effect of dietary polyamines on body growth of animals treated with AOM + DFMO and AOM alone were compared. The growth inhibitory effect of DFMO seen with 0 PA (group 4 versus 1) was reduced in the presence of 1 PA (group 5 versus 2), and abolished in the presence of 3 PA (group 6 versus 3).

Formation and growth of ACF

All AOM-treated animals (groups 1–6) developed ACF, while none of the animals in the other groups had ACF. Thus, polyamines alone do not induce ACF. In both experimental systems (AOM alone and AOM + DFMO), addition of polyamines to the diet resulted in a numerical, but not statistically significant increase in the formation (number) of ACF (Figure 1a). In contrast, dietary polyamines prominently promoted the growth of ACF (number of ACF_{≥3}, Figure 1b). In animals treated with AOM alone, a significant positive correlation ($r = 0.48$, $P = 0.043$) was demonstrated between the amount of polyamines added to the diet (0–3 PA) and the

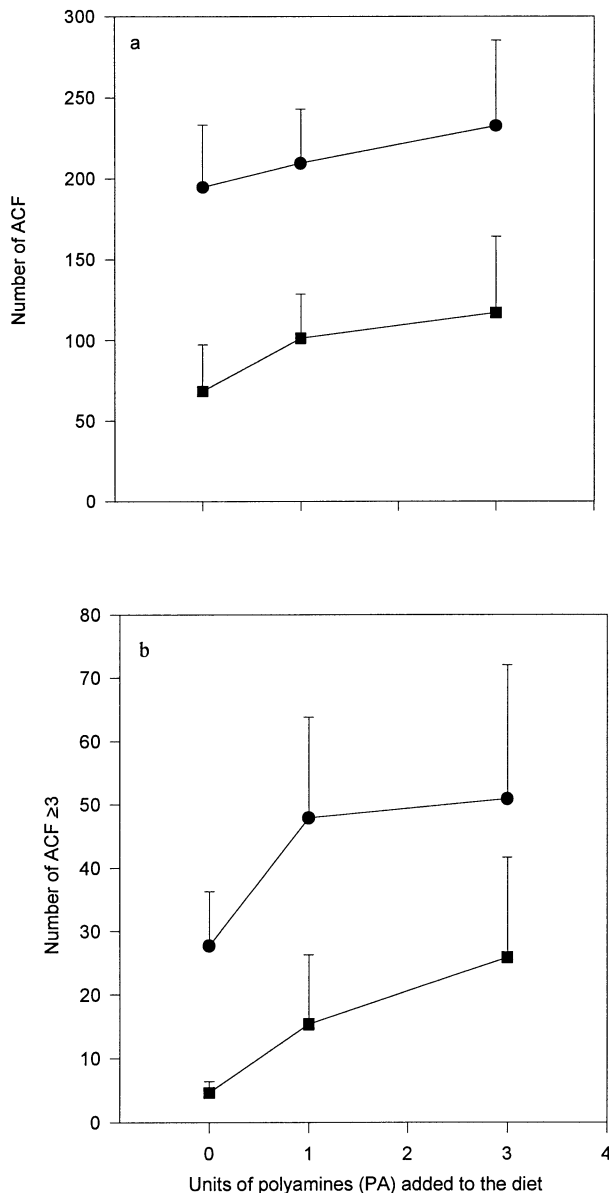


Fig. 1. The influence of dietary polyamines on the formation of ACF, expressed as the number of ACF (a) and on the growth of ACF, expressed as number of ACF ≥ 3 (b) in the colon of rats treated with AOM (●) and AOM + DFMO (■). Mean \pm SD is indicated. ($n = 6$). The basic AIN-76A diet (0 PA added) contains 50 nmol/g of putrescine, 10 nmol/g of spermidine and 1 nmol/g of spermine; the diet added 1 PA (see Materials and methods for definition) contains 280 nmol/g of putrescine, 261 nmol/g of spermidine and 31 nmol/g of spermine; the diet added 3 PA contains 740 nmol/g of putrescine, 763 nmol/g of spermidine and 91 nmol/g of spermine. The total number of ACF (\pm DFMO) (a) is not significantly changed by polyamines. The increased number of ACF ≥ 3 (b) seen after polyamine addition is statistically significant ($P < 0.05$); there is a significant dose-response correlation in animals treated with AOM alone (●, $r = 0.48$; $P = 0.043$) and in animals treated with AOM + DFMO (■, $r = 0.68$; $P = 0.002$). The inhibitory effect ($P < 0.05$) of DFMO on the total number of ACF (a) is not significantly altered by the addition of polyamines. The inhibitory effect ($P < 0.05$) of DFMO on the number of ACF ≥ 3 (b) is abolished with 3 PA.

number of ACF ≥ 3 ; the relative increase compared with the 0 PA group was 71% ($P = 0.04$) when 1 PA was added and 82% ($P = 0.04$) when 3 PA was added. In animals treated with AOM + DFMO, a more pronounced dose-response correlation was seen between the amount of polyamines in the

diet and the number of ACF ≥ 3 ($r = 0.68$, $P = 0.002$); the relative increase was as large as 420% ($P = 0.004$) with 3 PA.

The influence of dietary polyamines on the inhibitory effects of DFMO was examined by comparing the effects in the two experimental systems (DFMO + AOM versus AOM alone).

The inhibition of DFMO on ACF formation, which was 64% in the 0 PA group ($P = 0.002$, group 4 versus 1), was only slightly counteracted by dietary polyamines. In contrast, the DFMO-induced inhibition of ACF growth, which was 82% in the 0 PA group ($P = 0.002$, group 4 versus 1), was abolished in the presence of 3 PA (group 6 versus 3).

Polyamine levels in the colonic chyme

In spite of the fact that the basal AIN-76A diet (0 PA) contains only small amounts of polyamines, a considerable concentration of polyamines in the colonic lumen was observed (Table II). Feeding large amounts of dietary polyamines only resulted in a minor increase in luminal concentrations of spermidine and spermine whereas the luminal putrescine concentration did not change significantly (groups 2, 5, 6 and 7).

Neither DFMO nor AOM treatment alone influenced the luminal polyamine levels significantly (group 8 versus 9 and group 1 versus 9). However, when DFMO was given to the AOM-treated animals the levels of polyamines in the colonic chyme were significantly reduced. This effect was present in animals given the basal, 0 PA, diet (group 4 versus 1) as well as in animals given the polyamine supplemented diets (group 5 versus 2 and group 6 versus 3).

Discussion

Oral administration of polyamines has been reported both to stimulate the normal growth of intestinal mucosa and to reverse the inhibition of mucosal growth caused by DFMO (30), which is an inhibitor of polyamine synthesis. Dietary polyamines also counteract the inhibitory effect of DFMO on the growth of transplanted lung carcinoma cells (19). To the best of our knowledge, however, the present report is the first to demonstrate that dietary polyamines have a stimulatory effect on the growth of AOM-induced ACF, which are early lesions associated with colon carcinogenesis. This stimulatory effect of dietary polyamines was observed both when polyamine synthesis was enhanced with AOM (31) and when the synthesis was suppressed with DFMO.

The stimulatory effect of dietary polyamines on ACF was in the present study restricted to the parameter of ACF growth: the number of ACF ≥ 3 . The formation of ACF, expressed as the total number of ACF, was not significantly increased. The growth parameter, the number of ACF ≥ 3 , was differently affected in animals in the two experimental situations. In animals treated with AOM alone, the number of ACF ≥ 3 was only slightly and not significantly increased when the dietary polyamines were increased beyond the amounts present in the non-synthetic B&K standard rat chow (1 PA). In animals treated with AOM + DFMO, an additional effect on ACF growth took place when the polyamine content of the diet was further increased. This difference strongly implies that endogenously formed polyamines also have a stimulatory effect on the growth of ACF and that exogenous polyamines might compensate for a reduction in the endogenous production. Thus, both endogenous and exogenous polyamines influence colon carcinogenesis. The observation that the inhibitory effect of DFMO on the growth of ACF was overcome by feeding polyamines, implies that the polyamine content of the

Table II. Polyamine concentrations in the colonic content and in the experimental diets

Group	Treatment	Putrescine (nmol/g)		Spermidine (nmol/g)		Spermine (nmol/g)	
		Diet	Lumen	Diet	Lumen	Diet	Lumen
1	AOM + 0 PA	50	214 ± 97 ^a	10	330 ± 72 ^a	1	125 ± 43 ^{a,d}
2	AOM + 1 PA	280	272 ± 146 ^b	261	460 ± 77 ^{a,b}	31	260 ± 44 ^{a,e}
3	AOM + 3 PA	740	230 ± 99 ^c	763	359 ± 124	91	202 ± 91 ^f
4	AOM + DFMO + 0 PA	50	85 ± 26 ^a	10	232 ± 115	1	56 ± 16 ^{b,c,d}
5	AOM + DFMO + 1 PA	280	96 ± 36 ^b	261	267 ± 116 ^b	31	115 ± 43 ^{b,e}
6	AOM + DFMO + 3 PA	740	93 ± 26 ^c	763	306 ± 103	91	112 ± 24 ^{c,f}
7	Vehicle + 3 PA	740	163 ± 70	763	473 ± 154 ^c	91	194 ± 54 ^g
8	Vehicle + DFMO + 0 PA	50	181 ± 55	10	357 ± 99	1	70 ± 19
9	Vehicle + 0 PA	50	136 ± 20	10	227 ± 9 ^c	1	94 ± 3 ^g

Means within a column with the same superscript are significantly different ($P < 0.05$).

PA, units of polyamines, see Materials and methods for definition.

diet should be considered when chemopreventive agents that influence polyamine synthesis are tested. It is even possible that dietary polyamines might confound chemopreventive trials in general.

The fact that the polyamines added to the diet stimulated the growth of ACF (Figure 1) shows that dietary polyamines somehow gain access to the epithelial cells. The polyamines might reach the epithelial cells via the systemic circulation after being absorbed, and/or they might act locally on the epithelial cells during the absorption process. A systemic effect would most probably be due to absorption from the small intestine of polyamines that escape the extensive degradation in the intestinal mucosa and the liver (32–34). However, a fraction of dietary polyamines seems to be available also for colonic absorption since addition of 3 PA to the diet of otherwise untreated animals resulted in a significant increase in spermidine and spermine concentrations in colonic chyme (Table II, group 7 versus 9). Hessels *et al.* (20) also found the colonic concentrations of polyamines to be dependent of the diet. Their results were, nevertheless, partly contradictory to ours, since they found that fecal excretion of putrescine and spermidine, but not spermine was highly dependent on the type of diet and its polyamine content.

The polyamine concentration of the 1 PA diet was 5- to 31-fold higher than in the polyamine-poor diet (Table II). Nevertheless, when AOM-treated rats were fed the 1 PA diet, the colonic polyamines concentrations were only 1.3- to 2.0-fold higher than when the polyamine-poor diet was fed (AIN-76A). Further, also rats fed a polyamine-poor diet, had substantial concentrations of polyamines in the colonic content. A major part of the polyamines in the colonic content thus does not stem from the diet. Even though polyamines are formed by colonic bacteria (20), we believe that most of the colonic polyamines of rats given a polyamine-poor diet derive from cells that are normally exfoliated from the intestinal epithelium. This assumption is partly based on the fact that spermine was present in considerable amounts in the colon of rats given a diet virtually free of spermine, although it is known that spermine is produced only at a low rate by bacteria. The observation that the polyamine concentrations in the colon of AOM-treated rats fed a polyamine-poor diet were largely reduced by DFMO, a substance that does not affect the polyamine concentrations in bacteria (35), also supports this idea. A reduction in the amounts of polyamines from sequestered epithelial cells upon DFMO treatment is to be expected, since DFMO inhibits both the polyamine synthesis and the

cell proliferation. It is also remotely possible that the reduction in colonic polyamines in DFMO-treated animals could be because of increased absorption by polyamine depleted colonic epithelial cells.

The present study demonstrates that the addition of 1 PA, but not 3 PA, increased the specific growth rate of the rat. Similar results have been found in chicken, where feeding of 0.2% putrescine or 0.5% spermidine increased growth rates beyond that of controls, while larger supplements reduced growth (36,37). Thus, while moderate doses of polyamines seem to be growth promoting, higher doses may be toxic. Interestingly, it is also reported that prolonged intraperitoneal injection of very high doses of putrescine (300 µmol/kg every 2 days) reduced the incidence and numbers of AOM-induced tumors (38). *A priori*, a stimulatory effect of dietary polyamines on body growth should be expected to be mediated through the systemic circulation. This is not necessarily so. The polyamines, while in transit, may stimulate the growth of the intestinal mucosa, and thereby absorption of nutrients. Grants *et al.* (39) found that putrescine or ethylamine added to the diet increased the intestinal absorption of xylose. The importance of the polyamines for the growth of the intestine has also been demonstrated in experiments with calves and piglets fed soybean-based milk replacer. Addition of putrescine to the milk-replacer was shown to promote the development of the epithelium of the intestinal tract (39,40).

The present results warrant further studies to enlighten the role of dietary polyamines in experimental and human colon carcinogenesis as well as for the use of DFMO in chemoprevention.

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