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# Ability of Aberrant Crypt Foci Characteristics to Predict Colonic Tumor Incidence in Rats Fed Cholic Acid[1]

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## ABSTRACT

**Aberrant crypt foci (ACF) are putative preneoplastic lesions of colon cancer which are being utilized currently as a biological end point to evaluate the induction and modulation of colon carcinogenesis. In several previous short-term studies, the unexpected reduction of ACF by the reported colonic tumor promoter cholic acid (CHA) emphasized the need for a systematic evaluation of the growth of ACF in response to a tumor promoter.**

**The present study was conducted to determine if any characteristic(s) of ACF at various early stages of carcinogenesis would predict resulting tumor incidence in rats fed CHA. Male Sprague-Dawley rats received two injections of azoxymethane (20 mg/kg) and were fed either the AIN-76 diet or AIN-76 plus 0.2% CHA. The number, crypt multiplicity (number of crypts/focus), and size (area) of ACF were measured after 2, 8, 14, and 18 weeks in 5 rats/group. The number of ACF was lower ($P < 0.033$) in animals fed CHA at all time points. Average crypt multiplicity of ACF was greater ($P = 0.045$) from CHA-fed animals after 8 weeks compared to animals fed the AIN-76 diet. The average size of ACF was smaller in CHA-fed animals after 2 weeks and then tended to be larger than the sizes of the ACF from animals fed the AIN-76 diet. All remaining animals were killed after 18 weeks. Tumor incidence was higher ($P < 0.001$) in the CHA-fed group (63.2%) compared to the control diet group (29.4%). CHA-fed rats also had a higher number of tumors/tumor-bearing rat compared to control diet rats (1.96 *versus* 1.13). The main finding of this study is that the number of ACF at early time points did not predict tumor incidence. Crypt multiplicity was a consistent predictor of tumor outcome and should be measured in future studies using ACF as a biological end point. The CHA diet appears to provide a unique tumor-modulating environment that selectively enhances the growth of a smaller number of ACF leading to an increased number of tumors compared to a control diet. The mechanism(s) by which CHA mediates this effect warrants further investigation.**

## INTRODUCTION

ACF[3] were identified in the colons of carcinogen-treated animals and proposed to represent putative preneoplastic lesions by Bird (1). Evidence supporting the hypothesis that ACF are precursor lesions is accumulating (2–6). ACF (which have been referred to as microadenomas, precursor lesions, and dysplastic crypt foci) are currently being used as a bioassay to identify colon carcinogens (7, 8) and to study the modulation of colon cancer by various treatments (9–14). The ability of ACF to identify specifically colon carcinogens has been evaluated (2) and appears to be consistent (7, 8).

The response of ACF growth to tumor promoters and inhibitors has been variable. In modulation studies, ACF are initially induced by chemical carcinogens, animals are allocated to control and treatment groups, and the growth characteristics of the ACF are compared at various time points for evidence of the promotion or inhibition of colon cancer by treatment. Characteristics of ACF that can be quantified include the total number of ACF per colon, the size (area) of individual ACF, and the crypt multiplicity or number of crypts comprising each focus. The most common parameter measured is the number of ACF in all or part of the colon.

In several studies, the change in the number of ACF occurred in the direction expected; ACF were increased in the presence of known promoters (3, 14) and reduced in the presence of known inhibitors (9, 13). In contrast, research in our laboratory consistently demonstrated that feeding CHA, a reported colonic tumor promoter (15, 16), to rats treated with either azoxymethane or methylnitrosourea (17, 18) resulted in a decrease in the number of ACF. Zhang *et al.* (19) found that increased crypt multiplicity rather than enhanced number of ACF was associated with promotion. One study (20) found no correlation between the number or crypt multiplicity of ACF and the incidence of adenocarcinoma. One factor to be considered is that the ACF were measured at greatly varying time points (2–32 weeks after first carcinogen treatment). There has been no systematic evaluation of the response of ACF to a tumor promoter to determine (*a*) the time required to observe an effect, (*b*) the characteristic of ACF that best predicts tumor outcome, and (*c*) the consistency of this effect over time. Clearly, further understanding and characterization of the ACF bioassay in response to tumor modulation are needed. To establish ACF bioassay as a useful short-term assay, we must demonstrate that one or more of the characteristics of early ACF respond to modulation in a predictable manner under a wide variety of conditions.

The main objective of the present study was to determine the response of ACF in the early stages of colon carcinogenesis in rats treated with a colon carcinogen and then fed a diet containing 0.2% CHA. The effect of CHA on the proliferative status and colonic tumor-enhancing activity is well documented (15, 16, 21). The consistent ability of CHA to reduce the number of ACF opposes the expected response of ACF, suggesting that CHA may provide a very sensitive and stringent condition under which to test the validity of ACF characteristics as early predictors of colon carcinogenesis. If there is one characteristic that can be identified with this modulator, this characteristic may be the most consistent predictor under a wide variety of conditions.

## MATERIALS AND METHODS

**Animals.** One hundred seventy-eight male weanling Sprague-Dawley rats were obtained from Campus Breeding, Department of Animal Care, University of Manitoba, Winnipeg, Manitoba, Canada. Animals were housed in wire cages, 3 rats/cage, with a 12-h light and 12-h dark cycle. Temperature and humidity were controlled at 22°C and 50%, respectively. Animals were given laboratory chow and water *ad libitum* until initiation of the experiment. Animal care was in accordance with the guidelines of the Canadian Council of Animal Care.

**Carcinogen.** AOM (Sigma Chemical Co., St. Louis, MO) in sterile saline was injected s.c. at a dose of 20 mg/kg body weight. Animals were given the carcinogen on days 1 and 10. One week after the first injection, animals were still displaying symptoms of acute toxicity (weight loss and alopecia). To avoid

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[3] The abbreviations used are: ACF, aberrant crypt foci; AOM, azoxymethane; CHA, cholic acid.

early mortality, we delayed the second injection until day 10. Ten control animals were given sterile saline on both days.

**Diets.** Diets were formulated based on the composition of the AIN-76 diet (22, 23). The control diet contained 200 g vitamin-free casein, 3 g DL-methionine, 500 g dextrose, 150 g cornstarch, 50 g corn oil, 50 g cellulose, 10 g AIN-76 vitamin mix, 35 g AIN-76 mineral mix, and 2 g choline bitartrate/kg diet. All diet ingredients except corn oil (Mazola), cornstarch, and dextrose were from the United States Biochemical Corp. (Cleveland, OH). The CHA diet was the AIN-76 diet plus 0.2% CHA (Sigma) by weight. The purity of the CHA was approximately 98%, as determined by thin layer chromatography by the manufacturer.

**Study Design.** After the first injection on day 1, rats were randomly allocated to either the CHA diet (102 AOM-treated and 5 control rats) or the control diet (66 AOM-treated and 5 control rats). The number of animals assigned per group was based on power analysis of ACF data collected from short-term studies and predicted tumor outcome (24). Animals were allowed to eat the designated diets *ad libitum* starting on day 1. Initial and biweekly body weights were recorded. Food consumption was measured during the 3rd and 15th weeks.

**Aberrant Crypt Measurements.** Five AOM-treated animals each in the CHA and control diet groups were terminated at 2, 8, 14, and 18 weeks for measurement of ACF growth. Visualization and quantification of the number, size, and crypt multiplicity of ACF in the entire colon were conducted as described previously (1, 3). To determine size, an ocular grid was used to measure the approximate area occupied by the ACF as viewed at ×100. To determine crypt multiplicity, the number of crypts in each focus was recorded. The size of individual crypts was calculated by dividing the size by the crypt multiplicity of each ACF.

**Tumor Pathology.** Remaining animals were killed after 18 weeks as several animals began losing weight and showing fecal blood. Animals were coded to conceal the identity of the treatment group until histopathological evaluation was completed. The location, appearance, and dimensions of all suspicious lesions were recorded. Abnormal lesions included visible tumors and enlarged lymphoid aggregates. Each lesion was dissected out with 0.5 cm of surrounding mucosa and fixed in 10% neutral buffered formalin or 70% ethanol. Lesions were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The existence of neoplasms was identified microscopically and categorized as adenoma or adenocarcinoma. Adenocarcinomas showed marked nuclear pleomorphism and/or invasion of the submucosa. As described by Clinton *et al.* (25), histological evaluation of suspected lesions was necessary because palpatable exophytic lesions, including areas of abnormal hyperplasia and enlarged lymphoid follicles, were macroscopically indistinguishable from adenomas and would have increased tumor incidence falsely.

**Statistical Analysis.** SAS statistical software for microcomputers was used for all statistical analysis. Analysis of variance was used to analyze body weight data. Each time point was analyzed separately. A two-way analysis of variance (diet × time) was used to determine the effect of diet on multiplicity over all time points. The Student $t$ test for equal or unequal variance as appropriate was used to analyze parametric data at each time point. $\chi^2$ analysis determined the effect of diet on tumor incidence and distribution of ACF categorized by multiplicity. At 2 weeks, ACF were categorized as small (1 crypt/focus), medium (2 crypts/focus), or large (≥3 crypts/focus). At all other time points, categories were small (1–3 crypts/focus), medium (4–6 crypts/focus), and large (≥7 crypts/focus). In all cases, $P < 0.05$ was considered significant.

## RESULTS

The body weights of AOM-treated animals were lower ($P < 0.004$) than the saline-treated animals in both diet groups for the first 10 weeks (Table 1). Between 6 and 10 weeks, body weights of the saline-treated animals fed the CHA diet were also lower ($P = 0.009$) than saline-treated animals fed the control diet. After 12 weeks, no difference ($P > 0.05$) in body weights was observed among all groups. Throughout the experiment, there was no difference ($P > 0.05$) between the body weights of two diet groups in AOM-treated animals. Food consumption averaged 23.8 g/rat/day and was not significantly different between diet groups at the two time points analyzed (data not shown). Based on average food consumption, animals received approximately 47.6 mg CHA/day.

Table 1 *Body weights of rats fed the AIN-76 diet with or without CHA at various time intervals*

| | Treatment groups | | | |
|---|---|---|---|---|
| Wk | AIN-76 + CHA + AOM | AIN-76 + CHA −AOM | AIN-76 +AOM | AIN-76 −AOM |
| 0 | 120 ± 0.7[a] | 116 ± 1.4[a] | 121 ± 0.8[a] | 120 ± 2.7[a] |
| 1 | 132 ± 1.3[b] | 167 ± 3.4[a] | 137 ± 2.3[b] | 175 ± 4.8[a] |
| 2 | 173 ± 1.7[b] | 216 ± 10.1[a] | 182 ± 2.7[b] | 232 ± 7.8[a] |
| 4 | 290 ± 2.2[b] | 335 ± 7.2[a] | 302 ± 3.3[b] | 339 ± 10.5[a] |
| 6 | 365 ± 2.9[b] | 372 ± 22.9[b] | 371 ± 4.4[b] | 416 ± 19.3[a] |
| 8 | 422 ± 3.9[b] | 432 ± 12.7[b] | 436 ± 4.7[b] | 475 ± 16.8[a] |
| 10 | 461 ± 4.3[b] | 470 ± 15.1[b] | 480 ± 6.4[a,b] | 514 ± 26.3[a] |
| 12 | 502 ± 4.9[a] | 516 ± 25.1[a] | 518 ± 6.9[a] | 547 ± 25.7[a] |
| 14 | 523 ± 5.5[a] | 534 ± 22.8[a] | 542 ± 8.8[a] | 565 ± 28.2[a] |
| 16 | 548 ± 5.6[a] | 563 ± 24.4[a] | 570 ± 9.8[a] | 594 ± 29.4[a] |
| Final | 551 ± 6.1[a] | 569 ± 23.4[a] | 580 ± 11.0[a] | 598 ± 31.3[a] |

[a, b] Mean body weight (g) ± SE; values sharing a common superscript are not different from each other at each time period ($P > 0.05$). +AOM, animals given injections of AOM; −AOM, animals given injections of saline.

There were no ACF observed in rats receiving saline from either diet group. In AOM-treated rats, the number of ACF/colon was lower ($P < 0.033$) in animals fed CHA at all time points compared to rats fed the control diet (Fig. 1). The highest number of ACF occurred at 14 weeks in both diet groups.

Crypt multiplicity (or number of crypts per focus) was analyzed in several ways. When all ACF from each diet group at each time point were pooled (Table 2A), higher crypt multiplicity was demonstrated ($P < 0.0015$) in CHA-fed animals at 8, 14, and 18 weeks. When average crypt multiplicity was determined for each animal ($n = 5$ rats/diet group), crypt multiplicity was higher in CHA-fed animals at all time points but reached significance ($P = 0.045$) only at 8 weeks (Table 2B). Two-way analysis of variance demonstrated a significant effect ($P < 0.001$) of diet on mean multiplicity over all time points (data not shown). A decrease in the average crypt multiplicity occurred in both groups at 14 weeks. The decrease in average multiplicity of ACF at 14 weeks was due to the emergence of many new, small ACF, while the increase in the number of ACF at 14 weeks was due primarily to ACF of 1–3-crypt multiplicity. Crypt multiplicity was also analyzed by categorizing crypts as small, medium, or large, as described in "Materials and Methods." $\chi^2$ analysis was used to determine if the frequency of observations in each category differed because of the CHA diet. The distribution of small, medium, and large ACF in the two diet groups was different ($P < 0.002$) after 8, 14, and 18 weeks, with a higher percentage of ACF in the larger categories occurring in CHA-fed animals (Table 3). The percentage of small, medium, and large ACF in each animal was also calculated. The only significant differences detected when each category was compared at each time point were at 8 weeks (Table 4). Rats fed the CHA diet had a lower percentage of small ACF and a higher percentage of large ACF than rats fed the control diet. Two-way analysis determined a significant effect of diet over all time points, with a lower percentage of small ACF and a higher percentage of medium and large ACF in CHA-fed rats as compared to rats fed the control diet (Table 4).

The average size of ACF in CHA-fed animals was slightly lower after 2 weeks but became larger ($P = 0.001$) at 8 weeks (Fig. 2). No difference in the size of ACF occurred between diet groups at 14 and 18 weeks. Similar to multiplicity, the decrease in average size of ACF at 14 weeks was likely due to the emergence of new small ACF. The average size of crypts within the ACF foci was smaller for ACF in the CHA-fed rats at all time points but reached significance only at 18 weeks (Fig. 3).

In the 10 animals killed at 14 weeks for ACF measurements, 3 of 5 in the CHA diet group and 2 of 5 in the control diet group had

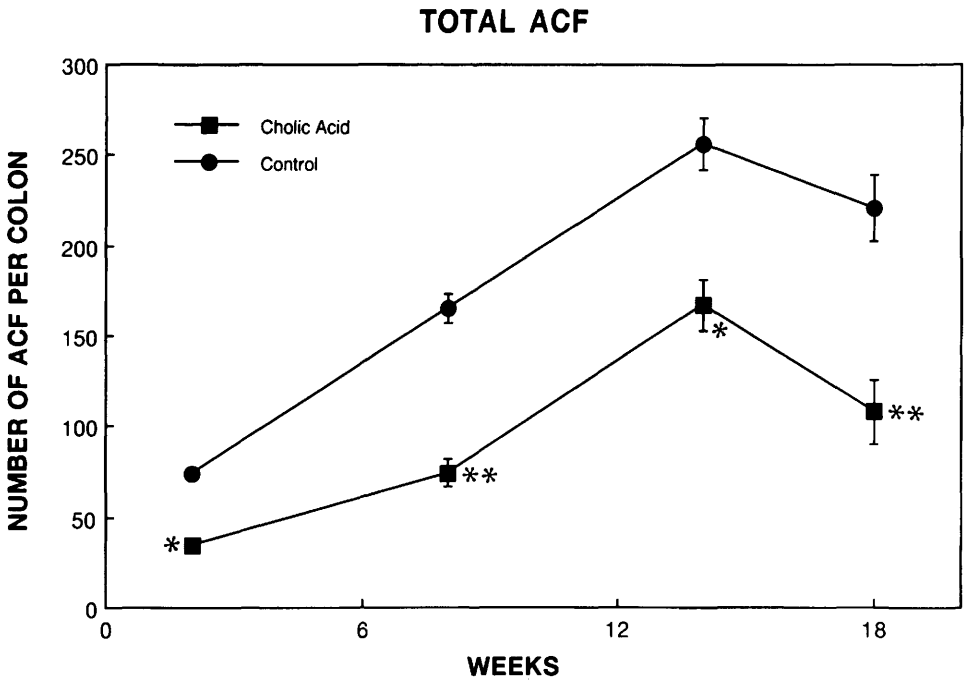


Fig. 1. Average number [mean ± SE (*bars*)] of ACF/colon in rats treated with azoxymethane and fed either the AIN-76 diet containing 0.2% cholic acid or the AIN-76 diet (control). Means with an *asterisk* are significantly different from corresponding control value: *, $P \leq 0.05$; **, $P \leq 0.01$.

Table 2 *Crypt multiplicity of ACF in rats treated with azoxymethane then fed either the AIN-76 diet containing 0.2% CHA or the AIN-76 diet alone*

| | A. Average | | |
|---|---|---|---|
| Wk[a] | AIN-76 + CHA[b] | AIN-76 | *P* |
| 2 | 1.28 ± 0.05 (172) | 1.19 ± 0.03 (374) | 0.0751 |
| 8 | 3.62 ± 0.01 (374) | 2.91 ± 0.05 (838) | 0.0001 |
| 14 | 2.73 ± 0.06 (836) | 2.50 ± 0.04 (1279) | 0.0015 |
| 18 | 3.81 ± 0.12 (540) | 3.30 ± 0.06 (1106) | 0.0001 |
| | B. Average of average | | |
| | AIN-76 + CHA[c] | AIN-76 | |
| 2 | 1.29 ± 0.05 | 1.19 ± 0.03 | 0.1380 |
| 8 | 3.63 ± 0.28 | 2.90 ± 0.11 | 0.0415 |
| 14 | 2.77 ± 0.15 | 2.46 ± 0.11 | 0.1360 |
| 18 | 3.87 ± 0.27 | 3.26 ± 0.22 | 0.1180 |

[a] Weeks on diet.
[b] Mean multiplicity of all ACF in 5 rats/group ± SE (number of ACF).
[c] Mean of mean crypt multiplicity in each rat ± SE ($n$ = 5 rats/diet group/time point).

tumors. After 18 weeks, no tumors were observed in animals receiving only saline in either diet group. Rats treated with AOM and fed the diet containing 0.2% CHA had a higher ($P$ = 0.001) overall incidence of colonic tumors compared to rats treated with AOM and fed the control diet (Table 5). The incidence of both adenocarcinomas ($P$ = 0.019) and adenomas ($P$ = 0.002) were higher in CHA-fed rats (Table 5). The number of tumors/tumor-bearing rat was higher in CHA-fed animals than in control diet-fed animals (Table 5). Tumor types included polypoid and sessile adenomas and adenocarcinomas, mucoid carcinoma in lymphoid follicles, and one undifferentiated small cell carcinoma. The size of tumors in the CHA-fed animals tended to be greater than in control-fed animals, but the difference was not significant (Table 5).

## DISCUSSION

The most important finding in this study is that the crypt multiplicity (or number of crypts/focus) and not the number of ACF was an early and persistent predictor of resulting tumor incidence. Rats treated with AOM and fed 0.2% CHA had a lower number of ACF throughout the study but developed more colonic tumors at 18 weeks than AOM-treated rats fed the control diet. Therefore, there was no time point at which the number of ACF predicted the enhanced tumor outcome due to feeding CHA but in fact predicted an inhibition of tumor development. In two previous studies the number of ACF increased in animals receiving a colonic tumor-promoting treatment (3, 14). McLellan and Bird (3) reported an increased number of ACF in rats fed a high fat diet, as well as an increased number of aberrant crypts/focus compared to rats fed a low fat diet. Shivapurkar *et al.* (14) measured ACF 12 weeks after carcinogen treatment and found a correlation between the number of ACF and the eventual tumor incidence in rats fed high and low risk diets. Therefore, although the number of ACF may correlate with eventual tumor outcome under some conditions, this parameter does not appear to respond to various tumor-promoting conditions in a consistent manner.

Throughout the experiment, the characteristic of ACF that was greater in CHA-fed rats predicting enhanced tumor outcome was the crypt multiplicity. This was demonstrated by comparing the distribution of ACF, categorized by crypt multiplicity in each diet group at each time point, and by comparing the average crypt multiplicity of all ACF from each diet group at each time point. A change in crypt multiplicity predicting tumor outcome occurred at an early time point (after 8 weeks) and persisted throughout the study. We have observed previously that the percentage of ACF categorized by crypt multiplicity is constant under various conditions of initiation, such as with varying doses (4) and varying number of injections[4] of carcinogen. Therefore, this characteristic appears to be specifically responding to the modulating effect of the CHA diet.

Consistent with our results, two other studies have found crypt multiplicity measured at one early time point to be a better predictor of tumor outcome than the number of ACF. Zhang *et al.* (19) reported that rats fed thermolyzed protein had higher crypt multiplicity of ACF and a higher number of large ACF after 14 weeks, with enhanced tumor incidence after 34 weeks of feeding. Pretlow *et al.* (26) recently reported a lower number of ACF with 4 or more crypts in rats treated with sodium phytate at 12 weeks and a lower tumor incidence at 36 weeks compared to rats not treated with sodium phytate.

It must be acknowledged that the difference between diet groups in crypt multiplicity was small at 2, 14, and 18 weeks, resulting in no

[4] Unpublished observations.

Table 3 *Percentage of ACF categorized as small, medium, or large based on multiplicity in the colons of rats treated with azoxymethane and fed either the AIN-76 diet with 0.2% CHA or the AIN-76 diet alone*

| Wk | Diet | ACF | | | | $P^b$ |
|---|---|---|---|---|---|---|
| | | Small[a] | Medium | Large | Total | |
| 2 | AIN-76 + CHA | 77.9[c] | 18.0 | 4.1 | 100 | 0.067 |
| | | (134) | (31) | (7) | (172) | |
| | AIN-76 | 85.8 | 11.2 | 2.9 | 99.9 | |
| | | (321) | (42) | (11) | (374) | |
| 8 | AIN-76 + CHA | 57.6 | 34.9 | 7.6 | 100.1 | 0.000 |
| | | (213) | (129) | (28) | (370) | |
| | AIN-76 | 74.3 | 23.0 | 2.6 | 99.9 | |
| | | (623) | (193) | (22) | (838) | |
| 14 | AIN-76 + CHA | 77.3 | 17.7 | 5.0 | 100.0 | 0.001 |
| | | (646) | (148) | (42) | (836) | |
| | AIN-76 | 79.8 | 18.2 | 2.0 | 100.0 | |
| | | (1020) | (233) | (25) | (1279) | |
| 18 | AIN-76 + CHA | 56.3 | 33.0 | 10.7 | 100.0 | 0.002 |
| | | (304) | (178) | (58) | (540) | |
| | AIN-76 | 65.6 | 27.9 | 6.5 | 100.0 | |
| | | (725) | (309) | (72) | (1106) | |

[a] At 2 weeks, ACF were categorized as small (1 crypt/focus), medium (2 crypts/focus), or large (≥3 crypts/focus). At all other time points, categories were small (1–3 crypts/focus), medium (4–6 crypts/focus), and large (≥7 crypts/focus).[b] $\chi^2$ analysis.[c] Percentage of the total number of ACF in each category (total number of ACF in 5 rats/diet group).

statistical difference when animal variation was included in the analysis, despite a 2-fold difference in tumor incidence. Animal variation in ACF development is to be expected, based on animal variation in susceptibility to tumor development. However, a large variation among animals reduces the power of ACF multiplicity to detect small changes due to the treatment. In this experiment, the use of several time points strengthens the evidence for an effect on crypt multiplicity due to diet. In studies in which only one time point was used, a larger number of animals would improve the sensitivity of this parameter as a bioassay. Using changes in the total population of ACF from each diet group to detect effects on multiplicity allows differences to be detected when a small number of animals are used, providing that investigators ensure that one or two animals do not skew the results.

The results of other studies demonstrate that the number and crypt multiplicity of ACF may not respond to a modulating environment in the same manner (9–11, 27). Because all of these studies were short-term, correlation with tumor incidence to determine which, if either, characteristic accurately reflected changes in tumor outcome was not possible. As mentioned earlier, the study by Hardman *et al.* (20) found no correlation between ACF number or crypt multiplicity and tumor outcome. There are several factors that may explain why the conclusions of these authors differ from the conclusions of this study and the studies by Zhang *et al.* (19) and Pretlow *et al.* (26): (*a*) Hardman *et al.* (20) utilized a multiple injection protocol, as compared to the one or two injection protocols used in the above studies; (*b*) Hardman *et al.* (20) measured crypt multiplicity in only 2 cm of the colon, whereas the above authors and this study measured ACF in the whole colon; (*c*) the ACF were measured only after 35 weeks of dietary intervention (coincident with adenocarcinoma development); therefore the value of the study by Hardman *et al.* (20) in addressing the predictive ability of ACF as an intermediate end point must be questioned.

Because the area or size of the ACF varies considerably for ACF of equal crypt multiplicity, measurement of this parameter may have been useful. However, differences in the size of ACF and the size of crypts within each ACF were either not consistent or not significantly different due to the cholic acid diet.

In summary, this study represents a systematic evaluation of the predictive ability of various characteristics of ACF in the early time

Table 4 *Average percentage of ACF categorized as small, medium, or large based on multiplicity in the colons of rats treated with azoxymethane and fed either the AIN-76 diet with 0.2% CHA or the AIN-76 diet alone*

| Weeks | Diet | Small ACF[a] | Medium ACF | Large ACF |
|---|---|---|---|---|
| 2 | AIN-76+CHA | 77.1 ± 3.7[b] | 18.6 ± 2.8 | 4.2 ± 1.3 |
| | AIN-76 | 85.5 ± 2.6 | 11.0 ± 2.5 | 3.4 ± 1.2 |
| 8 | AIN-76+CHA | 58.3 ± 5.1[c] | 34.5 ± 6.0 | 7.2 ± 1.0[c] |
| | AIN-76 | 75.0 ± 3.0 | 22.4 ± 2.7 | 2.6 ± 0.4 |
| 14 | AIN-76+CHA | 77.0 ± 2.7 | 18.1 ± 1.6 | 4.9 ± 1.4 |
| | AIN-76 | 79.8 ± 2.8 | 18.3 ± 2.6 | 2.0 ± 0.3 |
| 18 | AIN-76+CHA | 58.0 ± 5.4 | 31.9 ± 4.4 | 10.1 ± 2.2 |
| | AIN-76 | 66.2 ± 5.1 | 27.4 ± 3.6 | 6.4 ± 1.5 |
| Two-way analysis (*P*) | | | | |
| Diet | | 0.0057 | 0.0218 | 0.0059 |
| Wk | | 0.0002 | 0.0002 | 0.0090 |
| Interaction | | 0.4532 | 0.3714 | 0.6067 |
| % over all time points | | | | |
| AIN-76 + CHA | | 67.6 ± 3.15[d] | 25.8 ± 2.53 | 6.6 ± 0.98 |
| AIN-76 | | 76.6 ± 2.29 | 19.8 ± 1.91 | 3.6 ± 0.60 |

[a] At 2 weeks, ACF were categorized as small (1 crypt/focus), medium (2 crypts/focus), or large (≥3 crypts/focus). At all other time points, categories were small (1–3 crypts/focus), medium (4 6 crypts/focus), and large (≥7 crypts/focus).
[b] Mean of average percentage of ACF/category/rat ± SE ($n$ = 5 rats).
[c] Significantly different from rats fed the AIN-76 diet alone for 8 weeks ($P \leq 0.05$).
[d] Mean of average percentage of ACF/category at all time points ($n$ = 20 rats).

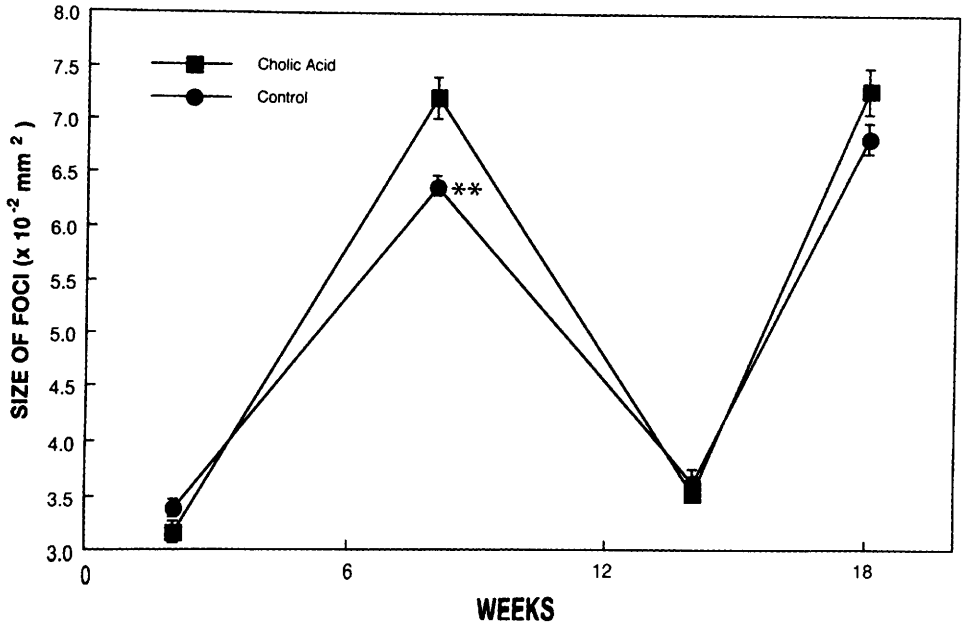


Fig. 2. Average size [mean ± SE (*bars*), area × $10^{-2}$ mm$^2$] of foci of aberrant crypts in rats treated with azoxymethane and fed either the AIN-76 diet containing 0.2% cholic acid or the AIN-76 diet (control). Means with an *asterisk* are significantly different; **, $P \leq 0.01$.

points of colonic tumor modulation based on resulting tumor incidence. The results from this and previous studies (19, 26) clearly demonstrate that the number of ACF alone cannot be used as a reliable indicator of promotion or inhibition of colon carcinogenesis. In the studies conducted to date, crypt multiplicity or number of crypts/focus appears to be a consistent predictor of tumor outcome and should be included in future studies using ACF as a biological end point. Studies with other modulators of colon carcinogenesis are needed to confirm the reliability of this parameter.

This study has confirmed earlier findings on the sequential growth of ACF. In both diet groups, an increase in number of ACF occurred up to 14 weeks, with a decrease occurring at 18 weeks. This resembles the early phase of the cyclic pattern of ACF growth reported in rats treated with one injection of dimethylhydrazine (28). These data support the contention that some ACF are transient lesions and either are eliminated or undergo remodeling to normal colonic crypts. In the resistant hepatocyte model of heptocarcinogenesis, only a very small subset of persistent nodules progress to development of carcinoma, with the remaining nodules remodeling to normal hepatocytes (29). Whether a similar phenomenon is occurring with ACF remains to be determined. In addition, the dramatic increase in small ACF at 14 weeks suggests that new ACF are continuing to form 12 weeks after the last carcinogen injection. McLellan *et al.* (28) similarly reported an increase in the number of small ACF 20–32 weeks after a single injection of dimethylhydrazine.

The other important issue that has been raised with these findings is that of the mechanism of modulation of colon carcinogenesis by CHA. It may be speculated that the decreased number of ACF in the cholic acid-fed animals may be due to an increased rate of elimination or remodeling as a result of increased cell proliferation. Feeding 0.2% cholic acid has been demonstrated previously to act as a mitogen for the colonic epithelium (21, 30) in animals not treated with a carcinogen. However, in studies using a multiple injection protocol, a mitogenic effect of cholic acid (15, 31) was not detected. We recently reported that feeding 0.2% cholic acid for 4 weeks stimulated colonic cell proliferation in animals treated with azoxymethane or methylnitrosourea (18) and resulted in a dramatic reduction in the number of ACF.

To our knowledge, no other modulator of colon carcinogenesis has been reported to significantly reduce the number of preneoplastic lesions, with a resulting increase in tumor incidence. The CHA diet appears to selectively enhance the growth of a small subset of the total

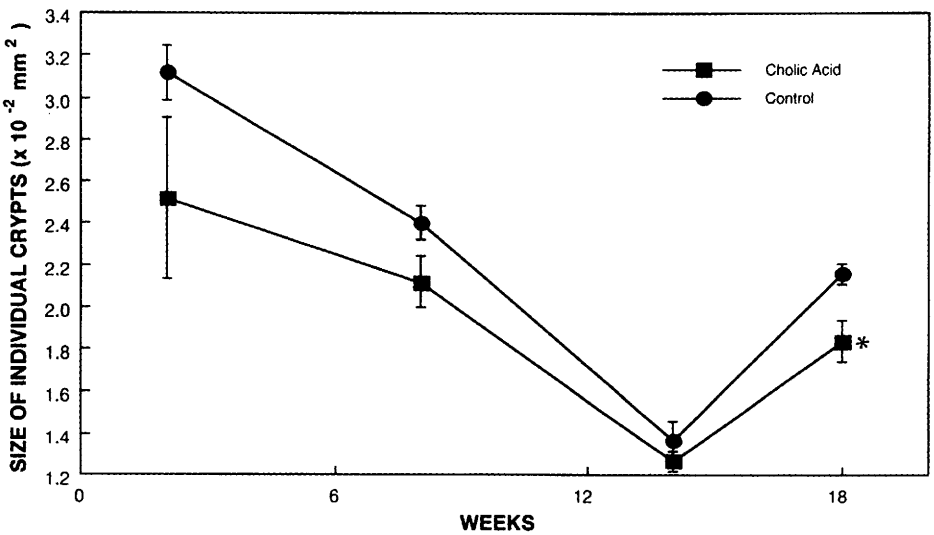


Fig. 3. Average size [mean ± SE (*bars*), area × $10^{-2}$ mm$^2$] of crypts within ACF in rats treated with azoxymethane and fed either the AIN-76 diet containing 0.2% cholic acid or the AIN-76 diet (control). Means with an *asterisk* are significantly different from corresponding control value: *, $P \leq 0.05$.

Table 5 *Tumor pathology in the colons of rats treated with azoxymethane and fed either the AIN-76 diet containing 0.2% CHA or the AIN-76 diet for 18 weeks*

| | AIN-76 + 0.2% CHA | AIN-76 | *P* |
|---|---|---|---|
| Rats with tumors | 55/87 (63.2%)[a] | 15/51 (29.4%)[a] | 0.001[b] |
| Rats with adenocarcinomas | 23/87 (26.4%)[a] | 5/51 (9.8%)[a] | 0.019[b] |
| Rats with adenomas | 42/87 (48.3%)[a] | 11/51 (21.6%)[a] | 0.002[b] |
| No. of tumors/tumor-bearing rat | 1.96 ± 0.29[c] (55) | 1.13 ± 0.09 (15) | 0.047[d] |
| Size of tumors (mm) | 3.65 ± 0.24[c] (108) | 3.00 ± 0.75 (17) | 0.338[d] |

[a] Numbers in parentheses, percentage.
[b] $\chi^2$ analysis.
[c] mean ± SE ($n$).
[d] Student's $t$ test.

population of ACF. Studies are in progress to determine if ACF in CHA-fed animals represent a selective subgroup of the heterogeneous population of ACF normally induced. Evidence for heterogeneity of ACF comes from observations of varying degrees of dysplasia within ACF of similar crypt multiplicity (28) and with various topographical characteristics (32).

The CHA diet provided a unique tumor-modulating environment which initially appeared to contradict the hypothesis that ACF may provide a useful model for evaluating the cancer-modulating effect of dietary components or chemopreventive agents. Nevertheless, the tumor-enhancing effect of the CHA diet was most valuable and led to the finding that crypt multiplicity was the characteristic which consistently predicted tumor incidence. If the CHA diet is promoting only a small, selected group of ACF, this model may prove useful in further studies to characterize ACF with a high potential to progress to malignancy.

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