MODULATING EFFECT OF CHOLIC ACID ON THE INDUCTION AND GROWTH OF ABERRANT CRYPT FOCI AND COLONIC TUMORS

by

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MODULATING EFFECT OF CHOLIC ACID ON THE INDUCTION AND GROWTH OF ABERRANT CRYPT FOCI AND COLONIC TUMORS

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Cholic acid, (CHA) a primary bile acid is known to enhance colon tumor incidence and modulate the number and growth of aberrant crypt foci (ACF) in the rat colon. Whether CHA enhances colon cancer as a co-carcinogen or tumor promoter remains uncertain. The purpose of this dissertation was to further assess the effect of CHA on colon carcinogenesis employing different experimental protocols. Number and growth of ACF and tumor incidence were used as end points in determining disease progression.

A study was carried out to determine if number and growth of aberrant crypt foci and tumor incidence can be modified by altering experimental protocol. Animals were injected with carcinogen, and randomly allocated to a diet containing 0.2% CHA immediately after carcinogen injection, or following a one week delay. Number, size and multiplicity (number of crypts/focus) was evaluated at three week intervals for 24 weeks. Animals fed cholic acid had consistently fewer aberrant crypt foci compared to control animals, however at later time points (week 15) aberrant crypt foci in the cholic acid groups had higher multiplicity. Animals receiving cholic acid diet immediately after carcinogen injection had significantly higher tumor incidence than control animals while the cholic acid-delay group exhibited an intermediate value (39.5%, 13.2%, 23.1% respectively, ($p \leq 0.05$).

An experiment was carried out to determine if cholic acid diet

exerts its ACF reducing effect at a late time point. The results showed that after 21 weeks of feeding control diet, three weeks of feeding cholic acid diet significantly reduced number of ACF (202 vs. 70, $P \leq 0.05$), specifically those with 1-4 crypts/focus . ACF with higher crypt multiplicity (≥5 crypts/focus) were not significantly altered.

Based on this information it was concluded that:

1) the 0.2% CHA diet exerts its effect at a very early time point and may be co-carcinogenic in nature

2) growth characteristics of ACF employing crypt multiplicity as an end point are an effective predictor of tumor incidence

3) ACF are biologically heterogeneous and those ACF with advanced growth features resist modulation by CHA diet

4) protocol is central to understanding and assessing the value of an exogenous agent as a cancer modulator.

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ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AC	aberrant crypt (s)
ACF	aberrant crypt focus (foci)
ANOVA	analysis of variance
AOM	azoxymethane
BrURD	bromodeoxyuridine
b.w.	body wieght
CH	crypt height
CHA	cholic acid
CHA-delay	cholic acid delay
CO	control
DMH	1,2-dimethylhydrazine
H & E	hematoxylin and eosin
i.p.	intra peritoneal
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	methylnitrosourea
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
s.c.	sub cutaneous
SEM	standard error of the mean

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Shirtliff, N., and Bird, R.P. Inability of Cholic acid, a known tumor promoter to enhance the number and growth of aberrant crypt foci (abs). Canadian Federation of Biological Societies Conference. 1993.

Shirtliff, N., Carr, I., and Bird, R.P. Variable effect of cholic acid on the induction and growth of aberrant crypt foci and colonic tumors in Sprague Dawley rats (abs). American Association of Cancer Research. 1993.

Chapter 1

INTRODUCTION

Carcinogenesis is recognized as a complex multistage process which can be modulated by endogenous and exogenous influences. More recent research has suggested that diet plays a key role in the development, progression, and possible prevention of the disease. Although dietary components have been shown to influence, the exact mechanism of action is not well known. This is complicated by the limited understanding of the carcinogenic process itself. In order to gain a better understanding of the mechanism by which promoters or inhibitors of colon cancer exert their effect, it is first essential to understand the disease process. The rodent model has been the most frequently used model in the study of cancer of the large bowel. Although this model has contributed greatly to the understanding of colon cancer, it's optimal use has been limited by the fact that various studies have utilized different approaches in attempting to induce and modulate the disease. Due to these inconsistencies it is difficult to determine when critical events in cancer development take place.

An understanding of the biology of the various steps of cancer development is important for successful intervention with exogenous agents. The aberrant crypt foci (ACF) model (Bird, 1987) provides a system which allows for the analysis of the precursor lesions as the disease is progressing. There is currently no *in vitro* system

that can be used to conduct cancer development studies. With the ACF model it is possible to analyze the disease process under various growth modulating conditions.

Bile acids have received a great deal of attention as colonic tumor promoters based on epidemiological (Hill, 1990) and animal model studies (Reddy et al., 1976; Reddy et al., 1977). It is proposed that bile acids promote colon tumor development by increasing cell proliferation. It is generally believed that increased cell proliferation leads to increased risk for cancer development. This concept has served as the basis for the search for agents which will reduce colonic cell proliferation or will alleviate bile acid induced mitogenic response in rodent colons. Cholic acid, a primary bile acid exerts a mitogenic response (Deschner, 1981) and enhances colonic tumor development when added to the diet (Cohen et al., 1980; McSherry et al., 1989) In previous studies, it was demonstrated that cholic acid reduced the number of ACF but enhanced colon tumor incidence when the diet was administered in conjunction with carcinogen treatment (Magnuson et al., in press). Furthermore it was demonstrated that a cholic acid containing diet exerted a growth inhibitory effect on ACF when the diet was administered four weeks after carcinogen treatment (Bird, 1990). These observations led to the proposal that cholic acid may exert different effects on the carcinogenic process depending on the time of its intervention.

Previous studies that have administered cholic acid diet in conjunction with carcinogen treatment show an increased tumor incidence. It is not clear however, whether cholic acid is acting as a co-carcinogen or as a distinct tumor promoter.

It was hypothesized that a cholic acid diet administered in conjunction with the carcinogen enhances tumor incidence by exerting a co-carcinogenic effect. Therefore, the main objectives of the present research were:

(1) to assess the effect of various levels of cholic acid on the proliferative indices of rat colon, and determine the length of time it takes for the effects to become apparent

(2) to assess the effect of a cholic acid containing diet on number and growth of ACF and tumor incidence when administered with, or one week after carcinogen administration

(3) to assess whether cholic acid diet is capable of modulating the growth and number of ACF exhibiting different growth characteristics

CHAPTER II

LITERATURE REVIEW

In order to put this research into the proper perspective, a review of the literature pertaining to the study of colon carcinogenesis, the relationship of diet to cancer, and the physiological significance of bile acids in normal and carcinogenic processes is necessary.

A. Introduction to Cancer

Cancer development is a multistep process characterized by the repeated selection of altered cell populations ultimately leading to the growth and development of an abnormal mass of malignant tissues (Farber, 1984). Cancer cells are characterized by three properties: 1) diminished or unrestrained growth, 2) invasion of local tissues, 3) spread or metastases to other parts of the body. Cells of benign tumors show diminished control of growth, but do not invade local tissues or spread to distant sites. Three distinct stages of cancer development have been defined: initiation, promotion, and progression (Farber 1984). Initiation occurs when the genetic component of cells are altered by a carcinogen which can be a chemical, viral, or radial energy. Promotion involves the expansion of initiated cells into focal lesions (nodules, papillomas, or polyps). Progression, the final,

often slower stage of cancer development, is the process by which focal proliferations undergo progressive growth resulting in malignant neoplastic cells. The three steps of cancer development are further composed of several substeps (Farber and Cameron, 1980).

B. Colon Cancer

1. Introduction

Colon cancer is one of the most prevalent forms of cancer in North America, and contributes significantly to premature death. An understanding of the pathogenesis of this disease and the factors which predispose one to increased risk, could reduce incidence and mortality. Thus research aimed at successful intervention must first establish cause and promotion.

Present knowledge of colon cancer indicates that the development and progression of the disease is complex and not well understood. The evolution of normal mucosa into colon cancer follows a multistep process involving proliferation of normal mucosa, adenoma formation, growth, and malignant transformation (Boone et al., 1992). There are a multiplicity of exogenous and endogenous factors that alone or in combination can result in the transformation of normal colonic cells into cancerous tissue. There is evidence however that environmental factors play a key role in the development of colon cancer (Neugut et al., 1993;

Zarkovic et al., 1993). Nutritional factors have been implicated in the etiology and prevention of colon cancer (Bruce, 1987; Burnstein, 1993) and the suggestion that by altering dietary intake, colon cancer incidence could be reduced by 80%-90% (Doll and Peto, 1981) has been widely accepted. Therefore, an understanding of the mechanisms by which colon cancer development can be modulated by diet and/or the biology of the disease is an important undertaking.

In view of the fact that diet can influence the carcinogenic process at various stages including metabolic activation and detoxification of carcinogens, DNA-repair responses, and selection and propagation of transformed cells with biological heterogeneity, it is important to define stage specific effects of dietary influences in the animal studies. Such an approach is vital for identifying a diet or dietary components capable of affecting the growth of neoplastic lesions rather than modifying the action of a carcinogen. This information can be applied meaningfully in chemoprevention or primary prevention of colon cancer.

2. Experimental Colon Carcinogenesis

The colon is an excellent organ for the experimental examination of carcinogenesis (Bruce, 1987). The colon is easily accessible and changes in morphology are easy to detect. As a result, all of the stages of cancer development can be examined and assessed. Detection of early lesions, examination of effects of modulators,

and final tumor incidence can all be quantified.

Two common approaches in the study of colon cancer and diet are currently used. Tumor incidence studies involve relatively large groups of animals maintained on a specific dietary regime for a long period of time (25-40 weeks). Tumorigenesis studies do not allow for the assessment of the stages of carcinogenesis nor do they allow for the examination of subtle changes caused by diet (Bird et al., 1989). Alternatively, risk markers can be used as the biological end point in cancer research using fewer animals for shorter periods of time (2-12 weeks). These studies however do not assess the disease itself, but assess the risk markers associated with the development of the disease (Bird et al., 1989). Ultimately a biomarker is needed that would allow for the assessment of the modulating effects of various dietary interventions. This biomarker would be representative of the actual biological lesion and more reflective of the disease process than risk markers.

3. Aberrant crypt foci

Bird et al., (1987) described a method for identifying and quantifying abnormal crypts in the colons of rodents treated with a colon carcinogen. It was hypothesized that these foci of aberrant crypts (AC) represent early precursor lesions of colon cancer. Further investigations supported this novel discovery (Bird et al., 1989).

It has been demonstrated that aberrant crypts possess atypical morphological characteristics and that these characteristics are more pronounced with time and/or increased multiplicity (McLellan et al., 1991b). Aberrant crypts have repeatedly been induced by colon carcinogens in a dose dependent and species dependent manner (McLellan et al., 1991a; McLellan and Bird, 1991; McLellan and Bird, 1988ab; Tudek et al., 1989). They grow in size and can be modified by diet (Archer et al., 1992; Caderni et al., 1991; Kendall et al., 1992; McLellan and Bird, 1988b; Stamp et al., 1993; Zhang et al, 1993; Zhang et al., 1992). The induction of aberrant crypts has been shown to be inhibited by well known inhibitors of colon cancer (Lam and Zhang, 1991; McLellan and Bird, 1991; Pereira and Khoury, 1991; Pretlow et al., 1992) further supporting the theory that aberrant crypts represent precursor lesions of colon cancer. Aberrant crypts have also been identified in human colonic tissue (Pretlow et al., 1992; Pretlow et al., 1991; Roncucci et al., 1992; Roncucci et al., 1991). In rats and humans, aberrant crypts may display varying degrees of dysplasia and histochemically-detectable altered enzyme activities (Pretlow et al., 1992).

Although seemingly simplistic, the aberrant crypt assay provides a sensitive approach for the study of early preneoplastic events in the colon and the stepwise assessment of the disease process. Once the colon is removed, the mucosal surface can be examined by light microscopy to identify aberrant crypts. Aberrant crypts can be

distinguished from normal crypts by their increased size, often elongated shape, and thicker epithelial lining (Bird, 1987). This method allows for direct evaluation and enumeration of aberrant crypts and of the effect of promoters or inhibitors. Number, size and multiplicity (number of aberrant crypts per focus) can be enumerated in each colon.

C. Diet and Cancer

1. Introduction

Epidemiological studies were first to indicate a link between diet and colon cancer. Clinical and experimental research followed to support this hypothesis (Reddy et al., 1980). Since Wynder et al.,(1969) and Burkitt (1971) first suggested that dietary factors, specifically dietary fat and fibre, may play a role in the etiology of colon cancer, a substantial amount of research has focused on these two nutrients. While dietary fibre is thought to play a protective role in cancer development, this research has revealed that not only the amount of fat but the type of fat, differing in saturation, is important in determining the effect on colon cancer development (Reddy, 1992). It is generally accepted that the promotional effect associated with dietary factors is increased cell proliferation (Jacobs 1988). Current research is focused on macronutrients such as calcium, vitamin D, (Newmark and Lipkin, 1992), protein (Zhang et al., 1992), carbohydrates (Caderni et al., 1991), and fat (Lapre et al., 1993).

2. Dietary Fat and Colon Cancer

Based on evidence generated by epidemiological studies, fat is most significantly associated with cancer development. While research in this field has been exhaustive, it remains unclear as to the exact mechanism of cancer development. The stage at which dietary fat appears to exert its effect is the post-initiation phase (Reddy, 1992; Reddy and Maeura, 1984). Currently, research suggests that dietary fat directly affects the amounts and types of compounds entering the colon. The high levels of free fatty acids in the ionized state can be both irritating and toxic to the colonic epithelium (Newmark and Lipkin, 1992). Resulting damage to the colonic epithelium would be compensated for by increased cell proliferation, a risk marker of colon cancer. The promotional effects of dietary fat associated with saturated fats depends on their fatty acid composition. Diets high in fish oils do not promote colon cancer, due to possible mediation on mucosal ornithine decarboxylase activity and/or prostaglandin synthesis (Reddy, 1992)whereas corn oil or safflower oil have marked tumor promoting activity (Reddy, 1986: Reddy and Maeura, 1984). Hill and associates (1971) were first to suggest the epidemiological correlation between diet and colon cancer might be explained by the involvement of bile acids. Although the exact mechanisms are not clear, several hypotheses relate high fat intake to the level of bile acids in the colon (Newmark and Lipkin, 1992).

D. Bile Acids

1. Introduction

Bile facilitates a number of critical functions in the gastrointestinal tract. As a secretory agent, bile promotes the secretion of water, lecithin, cholesterol and pigment. Bile is essential for the digestion and absorption of several substances. Bile helps to emulsify fat, assist pancreatic lipolysis, formulate mixed micelles and chylomicrons, and possibly stimulate re-esterification of fatty acids. Absorption of cholesterol, fat soluble vitamins, and other insoluble lipids is almost totally dependent on bile (Carey, 1982; Mayes, 1990). In the small intestine, bile salts release hormones, control cholesterol synthesis by mucosa, and possibly clean the mucosa and prevent bacterial growth (Heaton, 1972). In the colon, bile or bile acids may promote motility, and prevent excessive water reabsorption from the feces.

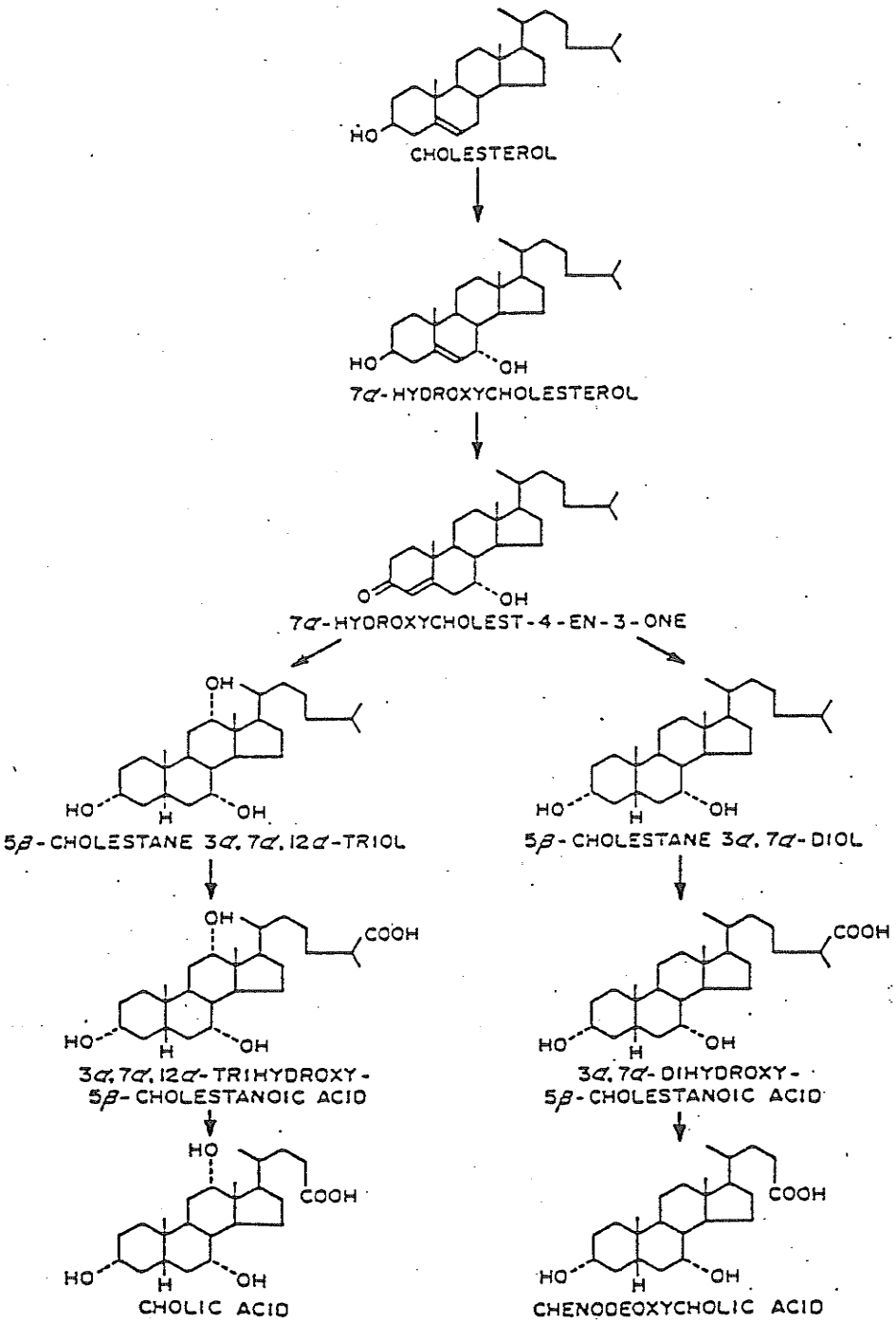
2. Biosynthesis and Secretion

Bile is a complex fluid containing fatty acids, cholesterol, bile acids, and phospholipid, as well as conjugated bilirubin, and trace amounts of protein and inorganic ions (Table 1). Bile acid is synthesized in the liver from cholesterol (Fig.1) via a series of reactions that include introduction of two hydroxyl groups at position 7 and 12, changing the orientation of the 3-hydroxyl group from beta to alpha, saturation of a double bond between C5 and C6,

Table 1 The composition of hepatic and gallbladder bile. (Taken from Hill, 1983.)

Substance	Hepatic	Gallbladder
Bile-acid conjugates	1.0%	11.5%
Cholesterol	0.1%	0.6%
Phospholipid (lecithin)	0.3%	3.5%
Bilirubin	0.1%	1.0%
Fatty acid	0.1%	1.0%
Protein	0.3%	0.4%
Inorganic salts	0.5%	0.8%
Bicarbonate	40 meq/l	10 meq/l
Chloride	100 meq/l	20 meq/l
Calcium	3 meq/l	6 meq/l
Potassium	6 meq/l	-
Sodium	150 meq/l	-
Magnesium	2 meq/l	0.2 meq/l

Fig. 1. Synthesis of bile acids. (Taken from Fielding and Fielding, 1985).



shortening the side chain by three carbon atoms, several oxidation steps and finally conjugation with taurine or glycine. Bile acids conjugated with glycine or taurine are referred to as bile salts. Approximately 98-99% of released bile acids are in the conjugated form. The major bile acids synthesized in the liver, referred to as the primary bile acids are cholic acid and chenodeoxycholic acid. Synthesis of bile begins in the endoplasmic reticulum and progresses through a complicated path as follows; cytoplasm, mitochondria, cytoplasm, endoplasmic reticulum, peroxisome, endoplasmic reticulum, and on to the bile duct. How the bile acid intermediates and the conjugated bile acids are transported through the cell is unknown, but several hypotheses suggesting the role of sterol binding proteins in the liver and intestine have been suggested (LeBlanc & Waxman, 1990: Lin et al., 1990).

Regulation of bile acid synthesis is controlled by several factors. The amount of bile acid synthesized in the body is dependent upon the amount returned to the liver via the enterohepatic circulation. This amount is normally slightly lower than that released from the liver so synthesis of bile acids is continuous. The first and rate limiting step in bile acid synthesis is the introduction of a hydroxyl group at the C-7 of cholesterol. This reaction is catalyzed by a unique cytochrome P-450 enzyme, cholesterol 7-hydroxylase. This enzyme represents a key point for removal of cholesterol from the body, and is thought to be regulated by several factors. Cholesterol derived from de

novo synthesis from plasma lipoproteins appears to be the main contributor of substrate for bile acid synthesis (Schwartz et al., 1982) and is largely responsible for the amount of bile acid produced. A secondary source of cholesterol is produced from hydrolysis of cholesterol esters. A high fat diet will also create increased demand for bile acid synthesis, as bile is essential for digestion of fat. Other factors that may cause an increase production of bile are increased thyroid hormone (Ness et al., 1990), and diurnal variation, (with maximal activity in the mid-dark phase), (Danielsson 1973).

3. Transport and Metabolism

The conjugated bile acids combined with the other components of bile are secreted from the parenchyma cells of the liver into the common bile duct. The bile is then stored in the gallbladder where it is concentrated by removal of water and electrolytes. Upon stimulation produced by fat entering the small intestine or the hormone cholecystokinin, bile is released into the duodenum. In the small intestine, bile acids act as detergents, forming mixed micelles with dietary fats and cholesterol and thereby promoting their uptake (Carey, 1982). Many of the compounds that constitute bile are reabsorbed from the gut lumen either by active transport (as with unconjugated bile acids), or by passive diffusion following deconjugation, or as part of the micellar phase during lipid absorption (as with cholesterol). Bacteria, in the gastrointestinal tract, that are responsible for the deconjugation

of bile acids, exist in minimal quantities in the small intestine, so most of the bile acids recovered from the terminal ileum are in the conjugated form. These compounds return to the liver via the portal blood, where they are reconjugated if necessary, and secreted once again in the bile. This process is known as the enterohepatic circulation (Fig.2) and is responsible for the reabsorption of approximately 95% of the intestinal bile acids. As little as 0.2-0.5 grams of bile acids are lost through the feces, and this amount is replaced by de-novo synthesis in the liver. The entire bile acid content (2 to 5 grams) recirculates through the body about 10 times per day.

The bile acids that are not reabsorbed in the small intestine are passed into the colon where the bulk of bile acid alteration takes place. The colonic flora is a rich mixture of both anaerobic and aerobic bacteria that act on biliary secreted compounds. The initial alteration that usually occurs is deconjugation of the bile salts. This step is followed by 7-dehydroxylation of cholic acid and chenodeoxycholic acid to produce deoxycholic acid and lithocholic acid, respectively. Secondary bile acids produced in the colon are absorbed into the enterohepatic circulation to a minimal extent. Deoxycholate is the chief secondary bile salt found in bile. It is the activity of these secondary bile acids that is of most importance in the development of colon cancer.
A wide variety of other secondary bile acids are produced as a result of oxidation, reduction, and epimerization (Table 2). Their

Fig. 2. The enterohepatic circulation. (Taken from Hill, 1983).

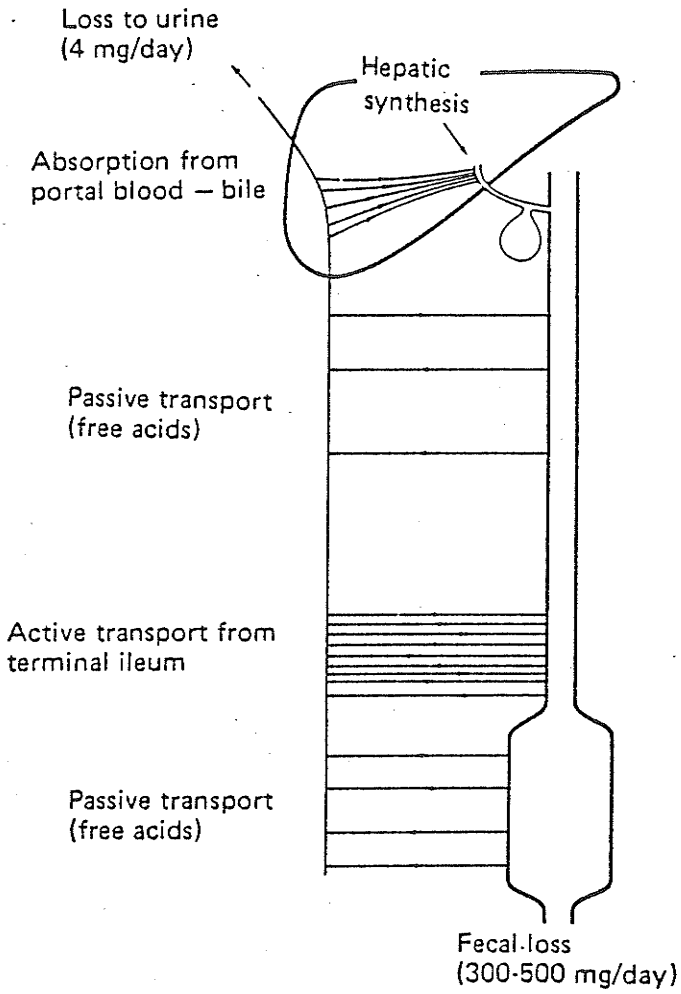


Table 2. Bile acids identified in human feces. (Taken from Carey, 1973).

Systematic name	Common name
3α,7α-Dihydroxy-12-keto-5β-cholanoic acid	
3α,12α-Dihydroxy-7-keto-5β-cholanoic acid	
3α,7β,12α-Trihydroxy-5β-cholanoic acid	
3β,7β,12α-Trihydroxy-5β-cholanoic acid	
3β,7α,12α-Trihydroxy-5β-cholanoic acid	
3α,7α,12α-Trihydroxy-5β-cholanoic acid	Cholic
3α,12α-Dihydroxy-5β-cholanoic acid	Deoxycholic
3,12-Diketo-5β-cholanoic acid	Dehydrodeoxycholic
3α-Hydroxy-12-keto-5β-cholanoic acid	
3β-Hydroxy-12-keto-5β-cholanoic acid	
12α-Hydroxy-3-keto-5β-cholanoic acid	
3α,12β-Dihydroxy-5β-cholanoic acid	
3β,12α-Dihydroxy-5β-cholanoic acid	
3β,12β-Dihydroxy-5β-cholanoic acid	
3α,7α-Dihydroxy-5β-cholanoic acid	Chenodeoxycholic
7α-Hydroxy-3-keto-5β-cholanoic acid	
3β,7α-Dihydroxy-5β-cholanoic acid	
3α-Hydroxy-7-keto-5β-cholanoic acid	7-Ketolithocholic
3α,7β-Dihydroxy-5β-cholanoic acid	Ursodeoxycholic
7β-Hydroxy-5β-cholanoic acid	
3α-Hydroxy-5β-cholanoic acid	Lithocholic
3β-Hydroxy-5β-cholanoic acid	Isolithocholic
3-Keto-5β-cholanoic acid	
5β-Cholanoic acid	Cholanic
3α,7α,12α-Trihydroxy-5α-cholanoic acid	Allocholic

concentration however, is minimal. It should be noted that some of these alterations may take place in the terminal ileum to a limited degree.

4. Bile Acids and Colon Cancer

There is mounting evidence that bile acids may play a role in the etiology of cancer of the large bowel. Circulating free bile acids in the colon are thought to be abrasive and toxic to the cells of the colonic epithelium. One of the resulting consequences is increased cell turn over, a condition associated with increased cancer risk. Studies have shown that certain dietary practices affect bile acid metabolism in ways that may have significance in intestinal carcinogenesis (Nigro and Campbell, 1976). Comparisons of feces from areas of high versus low incidence (Hill et al. 1971), found relatively more anaerobes and more strains able to 7-dehydroxylate cholic acid in samples from high incidence areas. It is assumed that this predisposes the individual to greater number of secondary bile acids in the colon, a suggested risk marker of cancer.

The effects of bile acids themselves on the colonic mucosa have been well documented. Hyperplasia (Deschner et al., 1981; Klurfeld et al., 1983; Williamson, 1978,), metabolic alterations and cell membrane disruptions (Dietschy, 1967; Vahouny et al., 1981), as well as stimulation of DNA synthesis (Friedman, 1981) have all been documented. Bile acids have also been shown to increase intestinal

permeability (Chadwick et al., 1979; Saunders et al., 1975) and increase absorption of DMH in the colon (Rose and Nahrwold, 1982). It has also been suggested that bile acids exert their effect by modifying mucosal immune function (Elitsur et al., 1990) or by activating PKC (Craven and DeRubertis, 1987; Huang et al., 1992). The interaction of bile acids with the metabolism of carcinogens (Turjman and Nair, 1981) has raised question as to their co-carcinogenic potential.

A number of in vitro studies have also been carried out which examine the relationship between bile acids and their potential role in the carcinogenic process. Single strand phage DNA incubated with bile acids show decreased transfection efficiency, suggesting that the bile acids damaged DNA (Cheah and Bernstein, 1990). Using C3H/10T1/2 cell line, Kawasumi et al., (1988) revealed that treatment with the carcinogen 3-methylcholanthrene (MCA) followed by, or preceded by, incubation with bile acids resulted in an increased number of transformed foci as compared to treatment with MCA alone. These results suggest that bile acids act not only as a promoter but a co-carcinogen under certain circumstances. Although the mechanisms are unclear, it has been suggested that the bile acids may increase cellular uptake of a carcinogen (Sugezawa and Kaibara, 1991) or influence cell-kinetic parameters, thereby enhancing intracellular fixation of the carcinogen and enhancing initiation (Kawasumi et al., 1988).

A multiplicity of studies have been carried out examining the possible cancer enhancing effect of bile acids. In tumor incidence studies, cancer is initiated with an injection of a carcinogen such as azoxymethane, N-Methyl-N-nitrosourea, or dimethylhydrazine followed by administration of a bile acid either through diet or intrarectal instillation. Dietary studies involving the administration of a bile acid generally show an increased tumor incidence (Cohen et al., 1980; McSherry et al., 1989; Sarwal et al., 1979). Similar dietary studies with or without a carcinogen reveal increased cell proliferation parameters (Cohen et al., 1980; Robblee et al., 1989; Bird et al., 1986). Several studies have utilized intrarectal or intragastric administration of a bile acid and again found increased tumor incidence (with carcinogen) or increased cell proliferation (with or without carcinogen), (Narisawa et al., 1974; Reddy et al., 1976; Reddy et al., 1977; Summerton et al., 1985). Cholestyramine, a bile acid sequestering agent, in theory would decrease tumor incidence. However, research has revealed that it has either no effect (Cruse et al., 1981) or it increases tumor incidence (Asano et al., 1975; Nigro et al., 1973). Using the aberrant crypt foci as an end marker in the study of colon cancer, Magnuson and Bird (1993) found that 0.2% cholic acid in the diet reduced the number and average size of aberrant crypt foci in the colon. Considering that aberrant crypt foci are putative preneoplastic lesions, this research raises questions as to the mechanism by which bile acids exert their effects and if they are promoters in all circumstances. It is essential to

evaluate protocol to determine if it plays a significant role in the outcome of cancer research.

E. Effect of Experimental Protocol in determining Cancer Outcome in Amimal Model Studies

1. Carcinogen administration

Several experimental protocols have been utilized in cancer research. Although sometimes conflicting, results are often considered conclusive. Despite the wide range of variation, no optimal protocol has been established, nor has the effect of different protocols on final outcome of the experiments been examined systematically. What may be a critical factor has largely been overlooked. It is essential to question the accuracy of comparing a one injection study to a multiple injection study. Would a series of carcinogen instillation each increase the potency of the previous instillation, thus blurring initiation and promotion? Furthermore, would the potency of a carcinogen not be affected when given concurrently with an exogenous agent? Finally, can subtle changes be accurately observed when excessive amounts of carcinogen are administered? Therefore, efficacy of a suspected tumor modulator must be altered by its ability to modify the carcinogen potency rather than the disease itself. By differentiating between an effect by the carcinogen and an effect by the disease process, we will be better able to focus intervention on either process. Since the nature and identity of

the carcinogen(s) involved in human colon cancer remain unknown, it is presently most logical to pursue means of preventing the disease process.

Cruse and associates (1981) carried out one study in which animals were injected with 20mg/kg body weight of dimethylhydrazine for 20 consecutive weeks, and concurrently administered 20mg/kg body weight of cholic acid, or 200mg/kg body weight of cholestyramine, or 22mg/kg body weight of aluminum hydroxide. Animals were maintained until death, whereupon tumor incidence evaluation revealed no promotional or inhibitory effect of any of the substances. It can be argued that the high dosage of carcinogen obscures any effect of the test agents. Clinton et al., (1988) examined the effect of ammonium acetate alone or in combination with sodium cholate on N-methyl-N'-nitro-N-nitrosoguanidine induced animals (4 injections). Intrarectal instillation of the test substances was delayed by two weeks following the final carcinogen administration. Tumor incidence revealed that ammonium acetate acted as a promoting agent but the bile acid did not. This brings into question the effect of delaying treatment for a substantial period following carcinogen injection. These two experiments using differing protocols come to conclusions contrary to other bile acid experiments previously cited. It is important, then, to examine other systems to try to determine if similar inconsistencies exist.

Extensive research in the field of liver carcinogenesis has revealed that animals initiated with liver carcinogens, develop foci of altered hepatocytes. These foci are considered to be preneoplastic lesions, and can be compared to the aberrant crypt foci found in the colon. The examination of the development and progression of these altered hepatocytes coupled with tumor incidence studies have provided a substantial amount of information about various carcinogens, promoters, and inhibitors of hepatocarcinoma. As with the colon however, results have not been conclusive. Bailey et al. (1987), found that indole-3-carbinol, a secondary metabolite from cruciferous vegetables, inhibits aflatoxin B_1 induced hepatocarcinogenesis in trout when given prior to and with the carcinogen, but promotes carcinogenesis when given continuously following initiation. Dashwood et al. (1991) found that the potential for indole-3-carbinol to promote carcinogenesis when fed prior to hepatic initiation is approximately as great as its potential to inhibit concurrent aflatoxin B_1 initiation. Dashwood et al. (1989), have suggested that differences in cancer incidence in the same species, using the same initiator/modulator pair but different regimen may be partially explained by the ability of the modulator to act as a blocking agent, preventing the carcinogen from binding with and damaging DNA.

An additional example noted in the liver model that is similar to the colon involves increased cell proliferation. Increased cell proliferation has been induced by various mechanism in an attempt

to correlate cell proliferation and cancer initiation in the liver. Partial hepatectomy or mitogens such as lead nitrate, nafenopin, cyproterone acetate can be used. Initiated rats exposed to increased cell proliferation that was mitogen-induced showed few hepatic enzyme altered islands, whereas partial hepatectomy, produced a large number of enzyme altered islands (Columbano et al., 1987; Ledda-Columbano et al., 1989). These results were consistent even when the carcinogen was administered at times corresponding to the various stages of cell cycle. It seems evident that increased cell proliferation is not solely responsible for these effects.

Despite years of research, it seems clear that the carcinogenesis process remains largely a mystery. Effort has been made to understand this process by a multiplicity of means. It is unfortunate that little is known about the stages of development of an initiated cell to a cancerous lesion. Once a protocol is established that allows examination of the stepwise development of cancer there will be greater opportunity to focus on how initiators, promoters, and inhibitors exert their effect.

Chapter III

MATERIALS AND METHODS

The materials and methods which were used in the various experiments are listed below.

A. Animals

Male sprague-Dawley rats were purchased from the Central Animal Breeding Facility, University of Manitoba. Rats were housed in stainless steel cages with wire mesh bottoms in groups of two or three. All animals were maintained on a 12-h light:dark cycle. Food and water were provided *ad libitum*. All animals were cared for in accordance to the guidelines of the Canadian Council on Animal Care.

B. Diets

In all studies, animals were acclimatized for approximately one week on Purina Laboratory Chow. This natural ingredient diet is made from ground corn, meat and bone meal, soybean meal, wheat middlings, ground wheat, ground oats, dehydrated alfalfa meal, dried milk product, brewers dried yeast, dried molasses, animal fat (with preservatives), iodized salt, dicalcium phosphate, and vitamin and mineral premixes. The composition of the Purina Laboratory Chow is presented in Table 3. The experimental regime consisted of a powdered AIN-76 semi-synthetic diet (Report of the

American Institute of Nutrition, 1984) with the exception that dextrose replaced sucrose as a source of carbohydrate. The composition of the AIN-76 diet is listed in Table 3. (See Appendix A for details of the vitamin and mineral mixture). Test diets were formulated by adding cholic acid (Sigma Chemical Co., St. Louis. MO) in various dosages (0.025, 0.05, 0.1, or 0.2 % by weight) to the AIN-76 diet.

Table 3. Percentages by weight composition of the diets.

AIN-76		LAB CHOW	
Ingredient	%	Ingredient	%
dextrose	50.0	NFE*	44.20
casein	20.0	protein	25.21
dextrin	15.0	ash	10.62
corn oil	5.0	moisture	9.28
alpha-cellulose	5.0	fat	6.37
AIN-76 mineral mix	3.5	crude fibre	4.32
AIN-76 vitamin mix	1.0		
DL-methionine	0.3		
choline bitartrate	0.2		

* NFE = nitrogen free extract - calculated carbohydrate portion

C. Preparation of the Carcinogen

The colon specific carcinogen azoxymethane (AOM) (Sigma Chemical Co., St. Louis, MO) was used in all experiments. The required concentration of AOM was prepared fresh in a saline solution prior to its use.

D. Body weights

Body weights (b.w.) of all animals were recorded at the start of each experiment and at appropriate intervals thereafter depending on the length of each study.

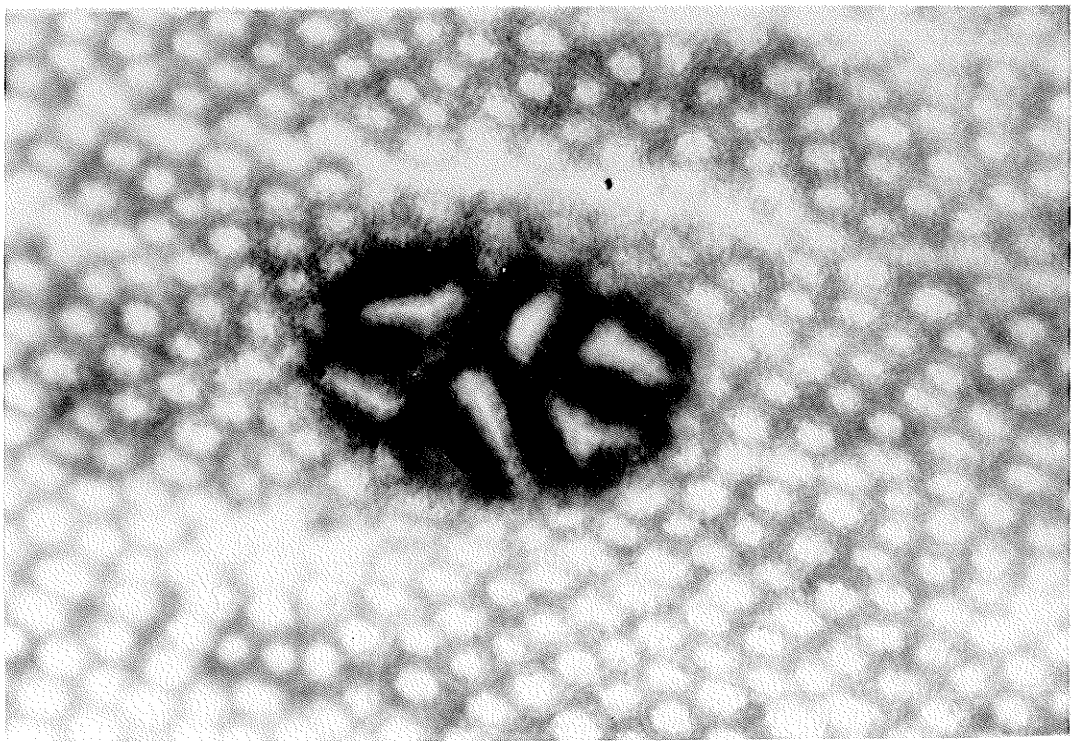
E. Preparation of the Colons

All animals were terminated by carbon dioxide asphyxiation. Upon termination, the colons were excised, flushed with phosphate buffered saline, slit open from caecum to anus, and fixed flat in 4% paraformaldehyde between filter papers. Colons which were to be analyzed for the presence of labelled cells were fixed in 70% ethanol (see G-2 below).

F. Identification and Quantification of Aberrant Crypt Foci

Colons were prepared and examined according to the method developed by Bird (1987). The colons were placed in a petri dish and stained for 15-30 minutes with 0.2% methylene blue (Sigma Chemical Co., St. Louis, MO) dissolved in phosphate buffered saline. The colons were then placed on a glass slide and the entire mucosal surface was examined under light microscopy at a magnification of 40x. Aberrant crypt foci (ACF) were distinguished from normal crypts by their increased size, elongated shape, more prominent epithelial lining, and larger pericryptal zone (the amount of area between bordering crypts) (McLellan and Bird, 1988). Typical ACF are shown in Figure 3. Number, size, and crypt multiplicity (number of crypts per focus) of ACF were determined for each colon by scanning

Figure 3. Topographical view of ACF consisting of various number of crypts.



the entire length of the colon beginning at the rectal end. Location of ACF was also recorded on a cm basis measured from rectal to cecal end. The size of ACF was assessed by placing a grid in the eyepiece of the light microscope. The number of squares on the grid that the focus occupied was then determined under magnification 100x.

G. Assessment of Proliferative Characteristics

1. Crypt height

Tissues were embedded in paraffin, sectioned longitudinally and mounted on slides. Prior to staining, the sections were de-waxed and rehydrated. The slides were then dipped in haematoxylin for 15 seconds and washed in running tap water. Excess stain was removed by decolorizing with 0.25% HCl in 70% alcohol. The blue colon was regained by washing in 2% sodium hydrogen carbonate. The slides were then stained with 0.5% eosin for five minutes, washed, and again dehydrated with alcohol. Nuclei were depicted by their blue-black coloration. The height of colonic crypts was measured by counting the nuclei lining the length of the crypts.

2. Bromodeoxyuridine labelled cells

Number of labelled cells in colonic crypts was determined with an immunohistochemical analysis. Animals received a s.c. injection of bromodeoxyuridine (BrUrd), 50 mg/kg b.w., (Sigma Chemical Co., St. Louis, MO) one hour prior to killing. The colon was removed and fixed in 70% ethanol for at least 24 hours. Tissues were embedded

in paraffin, sectioned longitudinally and placed on poly-l-lysine coated slides. These were baked overnight at temperatures no higher than 50-55^{0}C. The first step of the immunoperoxidase procedure involved deparaffinizing the slides with xylene followed by rehydration. The slides were immersed in 2N HCl for one hour and then in 0.1M $Na_2B_4O_7$. The slides were washed in phosphate buffered saline and treated with goat serum. Specimens were incubated with anti-BrUrd monoclonal antibody (diluted 1:40) for one hour, washed and treated with goat anti-mouse antibody. Mouse IgG peroxidase was then added, followed by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Cells that contained BrUrd were identified by the presence of a brownish pigment over nuclei. (See Appendix B for details of the procedure).

3. Mitotic figures

Assessment of mitotic figures was carried out by using metaphase arrest technique. Animals received a s.c. injection of colchicine (1mg/kg b.w.) two hours prior to termination. Following termination, the colon was removed and fixed in formalin for at least 24 hours. Sections were prepared and stained as outlined above in G-1 (crypt height). Metaphase figures were identified by their dark blue coloration and specific chromosomal arrangement around centromeres.

4. Proliferating cell nuclear antigen

Assessment of proliferating cell nuclear antigen (PCNA) was

determined with immunohistochemical analysis. Following termination, the colon was removed and fixed in 70% ethanol for at least 24 hours. Sections were prepared and stained in the same manner as described in G-2 but anti-PCNA monoclonal antibody was used. PCNA labelled cells were identified by the presence of brownish pigment over nuclei. (See Appendix B for details of the procedure).

H. Photography

All pictures were taken using Kodak Panatomic-X black and white or color film in a Nikon FX-35A camera which was attached to a Nikon microscope.

I. Statistical Analysis

Data analysis was performed by Student's t-test, CHI-square, or analysis of variance in combination with Duncan's multiple range test, using SAS software (SAS User's Guide, 1988). For all tests a probability of less than or equal to 5% ($p \leq 0.05$) was considered significant.

Chapter IV

EFFECT OF VARYING LEVELS OF CHOLIC ACID DIET ON CELL PROLIFERATION.

1. Introduction

A high fat diet and increased production of bile acids in the colonic lumen have been implicated in enhanced tumorigenic events in human and rodent colons (Hill, 1974; Narisawa et al., 1978; Nigro et al., 1976; Reddy et al., 1975; Reddy, 1975). The exact mechanism by which bile acids exert their effect is not known, however several possibilities have been suggested (Watabe and Bernstein, 1985; Wilpart et al., 1983). It is known that bile acids can damage the colonic epithelium and increase the proliferative activity (DNA synthesis and mitotic activity) of the colonic epithelium presumably as a repair response (Deschner et al., 1981; Lapre et al., 1993; Newmark et al., 1984; Nigro et al., 1976; Reddy et al., 1975; Reddy, 1975). A diet containing cholic acid (CHA), (0.2% by wt.) has been known to enhance colon tumor incidence (Deschner et al., 1981, Cohen, et al., 1980) and to induce a marked cytotoxic and hyperproliferative response in the rodent colons (Cohen et al., 1980). Limited information is available with respect to effect of feeding cholic acid at lower doses (<0.2%) on the proliferative status of the colonic epithelium in the rat. Therefore it was of interest to determine what the proliferative response would be when lower levels of bile acid was administered. This investigation was preliminary in nature and was

designed to examine the effect of varying levels of cholic acid on the proliferative status of the colonic epithelium. It was also of interest to determine if a distinct dose related response was evident when the lower levels of bile acids were used.

2. Materials and Methods

a. Animals

Male weanling Sprague-Dawley rats were used.

b. Study design

Following acclimatization, five animals were each randomly assigned to one of the following four dietary regimes: 0.0 %CHA (CO), 0.025% CHA, 0.05% CHA, 0.1% CHA (Figure 4). The animals remained on their respective diets for two weeks at which time all animals were terminated by carbon dioxide asphyxiation. Approximately 2 hours prior to termination, animals were injected with 1mg/kg colchicine i.p. to assess the proliferative status. One hour prior to termination, animals were injected with 50mg/kg BrUrd i.p. for determination of DNA synthesis. Colons were also stained by immunohistochemical methods to determine PCNA. Initial body weights were recorded and subsequent weighing took place at three day intervals.

3. Results and Discussion

Dietary intervention did not have a significant effect on body weight (Table 4).

Varying levels of CHA in the diet exerted a significant effect on

Table 4. Body weights[1] of animals consuming 0.0, 0.025, 0.05, or 0.1% CHA for two weeks.

	Dietary Regime			
	0.0% CHA (n = 5)	0.025%CHA (n = 5)	0.05% CHA (n = 5)	0.1% CHA (n = 5)
DAY 1	71.2 ± 4.1^{a}	82.8 ± 5.8^{a}	73.4 ± 5.2^{a}	73.8 ± 3.6^{a}
DAY 4	89.2 ± 3.4^{a}	102.2 ± 4.8^{a}	88.2 ± 5.8^{a}	88.6 ± 4.3^{a}
DAY 8	117.0 ± 4.3^{ab}	133.4 ± 6.2^{a}	115.2 ± 6.9^{b}	114.2 ± 4.7^{b}
DAY 11	136.2 ± 4.8^{b}	157.8 ± 8.1^{a}	135.6 ± 8.2^{b}	134.4 ± 5.1^{b}
DAY 14	167.8 ± 5.0^{a}	192.4 ± 9.7^{a}	168.2 ± 9.3^{a}	167.6 ± 6.1^{a}

[1]Values expressed as mean ± SEM. Means within a row not sharing a common superscript differ at $p \leq 0.05$.

some parameters of BrUrd labelling. Diets containing CHA significantly increased crypt height in the rectal region only (Table 5). BrUrd labelling index was significantly increased by CHA diet in the mid region of the colon (Table 5).

Diets containing CHA did not exert any consistent significant effect on mitotic activity of the colonic crypts. Nevertheless there was a tendency to increase crypt height in the rectal region and mitotic index in the mid region (Table 6).

PCNA labelling, a more novel approach to determining cell proliferation (Diebold et al., 1992; Lipkin, 1983; Richter et al., 1992; Risio et al., 1993; Sheneber et al., 1993) revealed an increase of activity corresponding to amount of CHA in the diet with significant differences in number of labelled cells and size of proliferative zone (Appendix D).

The main findings of this study are that feeding a diet containing low levels of cholic acid has a varied effect on the cell proliferation parameters in the colons of rats. Differences in proliferative activity between different regions of the colon are frequent and can be ascribed to the different conditions that exist in each region. Because the bile acids are more tightly bound in the rectal region, they are unable to exert as much effect here. Their effect is much more evident in the mid region of the colon. In the mid region, there appears to be a slight increase of all

Table 5. BrUrd labelled cells[1] in colons of animals consuming 0.0, 0.025, 0.05, or 0.1% CHA for two weeks.

Rectal end[2]

DIET	Crypt Height	# Labelled Cells	Labelling Index	Size of Prolif. Zone
0.0% CHA	31.2 ± 1.4^{b}	2.6 ± 0.4^{a}	4.2 ± 0.7^{a}	7.9 ± 0.9^{b}
0.025%CHA	35.2 ± 1.6^{a}	2.8 ± 0.3^{a}	4.0 ± 0.4^{a}	8.4 ± 0.8^{b}
0.05%CHA	38.2 ± 1.3^{a}	2.9 ± 0.4^{a}	3.8 ± 0.5^{a}	11.3 ± 1.2^{a}
0.1%CHA	36.1 ± 1.3^{a}	2.5 ± 0.3^{a}	3.5 ± 0.3^{a}	9.3 ± 0.4^{ab}

Mid section[3]

0.0%CHA	37.5 ± 1.93^{a}	2.9 ± 0.5^{b}	3.6 ± 0.5^{b}	12.1 ± 1.5^{a}
0.025%CHA	39.3 ± 1.2^{a}	4.7 ± 0.4^{ab}	6.3 ± 0.4^{a}	13.5 ± 0.8^{a}
0.05%CHA	39.5 ± 2.2^{a}	4.7 ± 0.9^{ab}	6.0 ± 0.9^{a}	13.4 ± 2.8^{a}
0.1%CHA	39.6 ± 1.2^{a}	5.6 ± 0.8^{a}	7.6 ± 0.8^{a}	14.3 ± 1.0^{a}

[1]Values expressed as means ± SEM. Means within each column not sharing a common superscript are significantly different ($p \leq 0.05$).
[2]cm 1-2 from the rectal end of the colon.
[3]cm 5-6 from the rectal end of the colon.

Table 6. Mitotic index[1] in colons of animals consuming 0.0, 0.025, 0.05, or 0.1% CHA for two weeks.

Rectal end[2]

DIET	Crypt Height	# Mitotic Cells	Mitotic Index	Size of Prolif. Zone
0.0% CHA	33.8 ± 0.9^{a}	2.2 ± 0.3^{a}	3.3 ± 0.5^{a}	9.5 ± 0.9^{a}
0.025%CHA	35.9 ± 1.6^{a}	2.1 ± 0.2^{a}	8.1 ± 5.1^{a}	10.8 ± 1.0^{a}
0.05%CHA	38.1 ± 2.0^{a}	2.8 ± 0.8^{a}	3.6 ± 0.8^{a}	10.1 ± 1.8^{a}
0.1%CHA	37.5 ± 0.9^{a}	2.2 ± 0.4^{a}	3.1 ± 0.6^{a}	9.0 ± 0.8^{a}

Mid section[3]

0.0% CHA	39.4 ± 1.7^{a}	2.9 ± 0.5^{ab}	3.7 ± 0.7^{ab}	14.8 ± 2.1^{a}
0.025%CHA	37.5 ± 1.3^{a}	2.5 ± 0.3^{b}	3.4 ± 0.4^{b}	14.6 ± 1.6^{a}
0.05%CHA	40.4 ± 1.0^{a}	3.9 ± 0.7^{ab}	4.9 ± 0.9^{ab}	17.8 ± 1.4^{a}
0.1%CHA	39.0 ± 1.2^{a}	4.5 ± 0.5^{a}	5.7 ± 0.6^{a}	16.2 ± 1.0^{a}

[1]Values expressed as mean ± SEM. Means within a column not sharing a common superscript are significantly different ($p \leq 0.05$).
[2]cm 1-2 from the rectal end of the colon.
[3]cm 5-6 from the rectal end of the colon.

parameters of cell proliferation as level of bile acid is increased. Although not all differences are significant, it is interesting to note that a dose related response seems to exist. Results of the different types of measurement may differ partially because each measures a different phenomenon. Mitotic index measures actual cell division, while BrUrd measures number of cells in S-phase. It is important to note that these parameters do not always increase concurrently.

This information is valuable in that it allows us to examine how administration of amounts of cholic acid less that 0.2% in the diet affect the colonic epithelium when administered on a short term basis. This information provides us with some insight into the effect of bile acids that has not been previously provided by studies that use higher amounts of bile acids when measuring proliferative status.

Chapter V

EFFECT OF DIETARY CHOLIC ACID ON CELL PROLIFERATION AT EARLY TIME POINTS

1. Introduction

There is increasing evidence that bile acids exert a cytotoxic effect on colonic epithelium. In light of controversial literature as well as information generated in a previous study, it is clear that the effects are not well defined nor consistent. Furthermore evidence from a previous study revealed that diets containing low doses of CHA (<0.2%) do not effect cell proliferation as significantly as diets containing 0.2% CHA (Bird et al., 1986; Robblee et al., 1989). Considering that a diet containing 0.2% CHA will likely cause an increase in cell proliferation parameters, it was of interest to determine when this response becomes evident, and if the response remains consistent or changes with time.

The main objective of this study was to assess the effect of feeding a diet containing 0.2% CHA, on the proliferative indices of the colon at three day intervals over a 12 day period. The information generated from this study will determine the period of time required before the effect of bile acid becomes evident. Secondly this information will determine if the bile acid effect remains consistent over time or if the effect may be overcome.

2. Materials and Methods

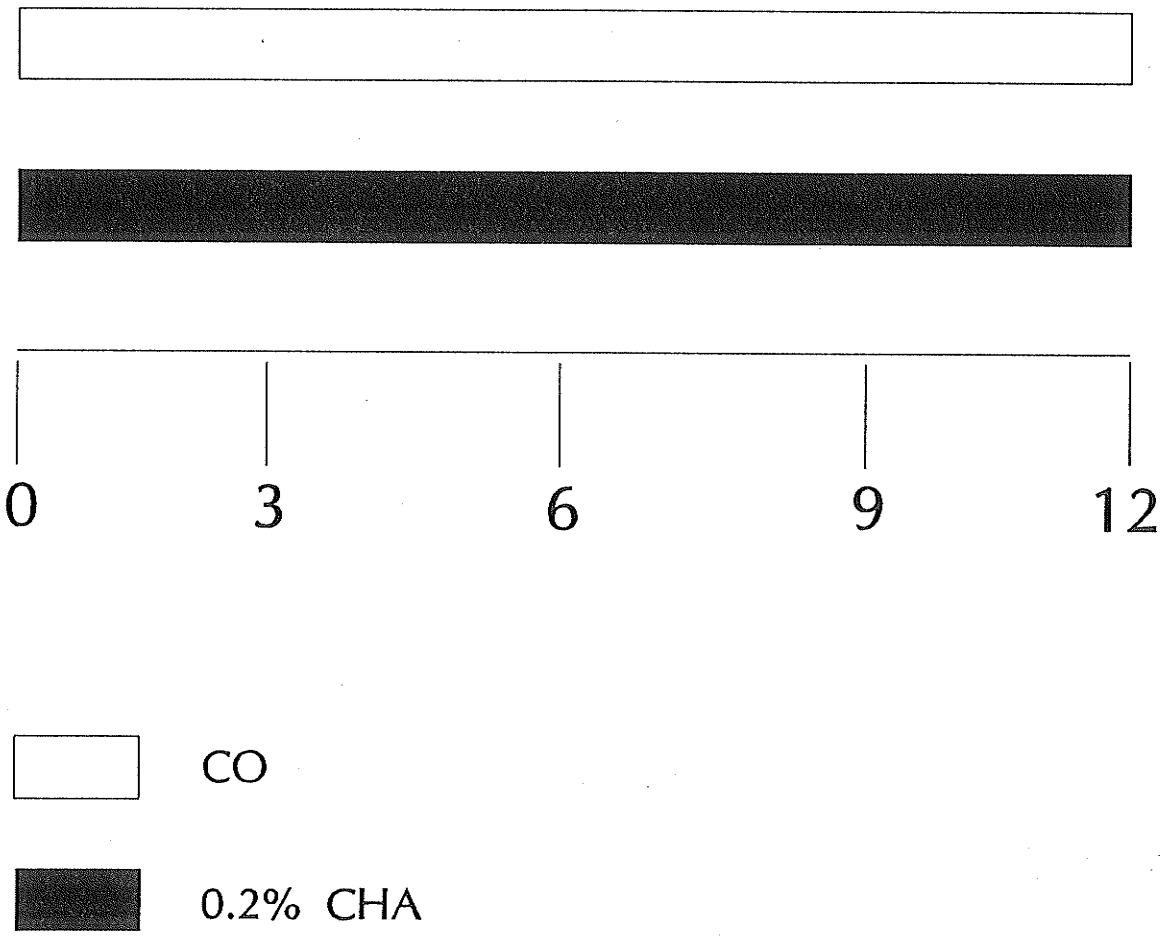
a. Animals

Male Sprague-Dawley rats weighing approximately 109.4 g. were used.

b. Study Design

The study design is schematically represented in Figure 5. following acclimatization, 24 animals were randomly assigned to one of two treatment groups. Twelve animals were assigned to the AIN-76 control diet (CO group) and 12 animals received the AIN-76 diet supplemented with 0.2% cholic acid (CHA group). Three animals per group were terminated at 3 day intervals at which time all animals were injected with colchicine and bromodeoxyuridine for assessment of proliferative features.

Figure 4. Schematic representation of experimental protocol for rats given diets containing 0.0% or 0.2% CHA for 12 days.



3. Results

The number of BrUrd labelled cells in the rectal and mid sections of the colon are represented in Table 7 and Table 8, respectively. In the rectal section, there is a significant increase in the number of labelled cells and labelling index at 9 days compared to control animals, yet these differences are not apparent at 12 days. In contrast, in the mid section the same parameters decrease at day 12 yet remain significantly higher than control values. Size of proliferative zone is significantly increased in the mid section at 9 and 12 days compared to control values. Topographical observation of the CHA group compared to control at day nine is presented in Figure 6. Morphological differences such as number and location of labelled cells, number of goblet cells, and position of nuclei are evident.

The proliferative indices as measured by mitotic index are presented in Table 9 and Table 10. In the rectal section there is a significant increase in crypt height compared to control at day 12 only (Table 9). There is a significant increase in number of mitotic cells and mitotic index at day 9, but again this difference is not evident at day 12 (Table 9). In the mid section, crypt height is elevated at days 9 and 12 (Table 10). Number of mitotic cells and size of proliferative zone is increased at day 9 only (Table 10). Topographical view of CHA and control group (Figure 7) depicts differences between the two groups such as number of mitotic cells and size of proliferative zone are evident.

Table 7. BrUrd labelled cells[1] in the rectal section[2] of colons from rats fed 0.0% or 0.2% CHA for 12 days.

Diet/ Time	Crypt Height	# Labelled Cells	Labelling Index	Size of Prolif. Zone
CO-3	38.0 ± 2.8^{b}	1.6 ± 0.2^{c}	2.1 ± 0.3^{c}	7.3 ± 1.4^{bc}
CO-6	35.7 ± 2.3^{b}	1.6 ± 0.1^{c}	2.3 ± 0.2^{c}	6.6 ± 0.4^{c}
CO-9	35.4 ± 0.4^{b}	2.1 ± 0.1^{c}	3.1 ± 0.1^{bc}	8.6 ±0.9^{abc}
CO-12	34.4 ± 1.3^{b}	2.7 ± 0.3^{bc}	4.0 ± 0.5^{bc}	9.3 ± 1.3^{abc}
CHA-3	38.6 ± 1.5^{ab}	2.1 ± 0.4^{c}	2.8 ± 0.7^{c}	7.4 ± 1.3^{bc}
CHA-6	37.4 ± 0.4^{b}	3.1 ± 0.8^{bc}	4.2 ± 1.1^{bc}	11.0 ± 1.8^{abc}
CHA-9	38.9 ± 0.9^{ab}	6.4 ± 0.6^{a}	8.3 ± 0.6^{a}	14.0 ± 2.0^{a}
CHA-12	43.8 ± 2.8^{a}	4.3 ± 0.8^{b}	5.2 ± 1.4^{b}	12.3 ± 3.0^{ab}

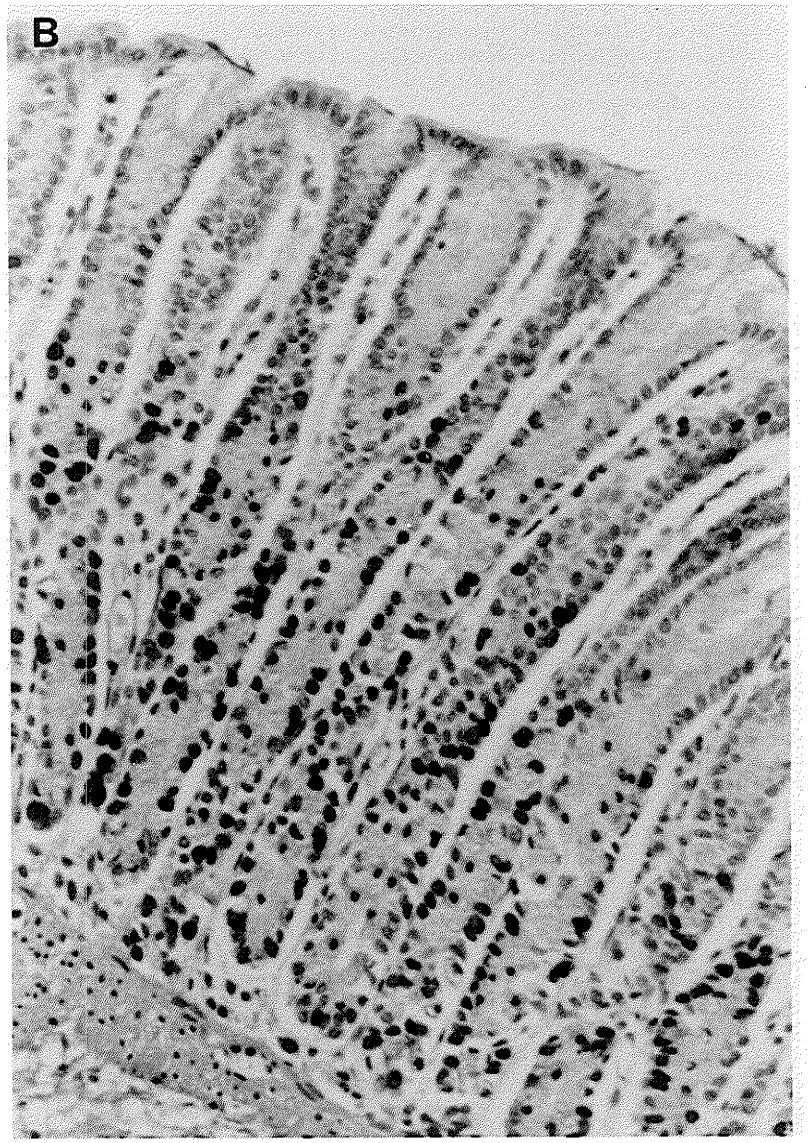
[1]Values expressed as mean ± SEM. Values within a column with different superscripts differ at $p \leq 0.05$.
[2]cm 1-2 from rectal end of the colon.
CO-3 = 0.0% CHA diet for 3 days, etc.
CHA-3 = 0.2% CHA diet for 3 days etc.

Table 8. BrUrd labelled cells[1] in the mid section[2] of colons from rats fed 0.0% or 0.2% CHA for 12 days.

Diet/Time	Crypt Height	# Labelled Cells	Labelling Index	Size of Prolif. Zone
CO-3	38.2 ± 1.9^{abc}	2.2 ± 0.1^{c}	2.9 ± 0.3^{c}	10.1 ± 1.9^{b}
CO-6	36.7 ± 1.1^{bc}	3.1 ± 0.6^{c}	4.3 ± 0.7^{c}	11.7 ± 0.7^{b}
CO-9	36.6 ± 1.6^{bc}	2.8 ± 1.6^{c}	3.8 ± 0.1^{c}	9.5 ± 0.6^{b}
CO-12	34.2 ± 0.6^{c}	3.1 ± 0.5^{c}	4.5 ± 0.8^{c}	10.8 ± 1.1^{b}
CHA-3	37.3 ± 1.7^{bc}	2.2 ± 0.4^{c}	3.0 ± 0.4^{c}	11.1 ± 2.9^{b}
CHA-6	41.7 ± 2.7^{ab}	4.0 ± 0.8^{c}	4.9 ± 0.7^{c}	13.9 ± 1.5^{ab}
CHA-9	43.0 ± 4.3^{ab}	11.3 ± 1.8^{a}	12.9 ± 0.8^{a}	19.4 ± 3.3^{a}
CHA-12	44.7 ± 0.6^{a}	7.3 ± 0.6^{b}	8.2 ± 0.5^{b}	18.4 ± 1.4^{a}

[1]Values expressed as mean ± SEM. Values in each column with different superscripts differ at $p \leq 0.05$.
[2]cm 5-6 from the rectal end of the colon.
CO-3 = 0.0% CHA diet for 3 days etc.
CHA-3 = 0.2% CHA for 3 days etc.

Figure 5. Longitudinal sections of colonic crypts of rats depicting BrUrd labelled cells. (a) control animal depicting large number of goblet cells. Note the nuclei are rounded and the labelled cells exist in the bottom one third of the crypt (40X). (b) Animals fed 0.2% CHA for 9 days. Note the reduced number of goblet cells, nuclear crowding, elongation of crypts, and expansion of the proliferative zone (40X).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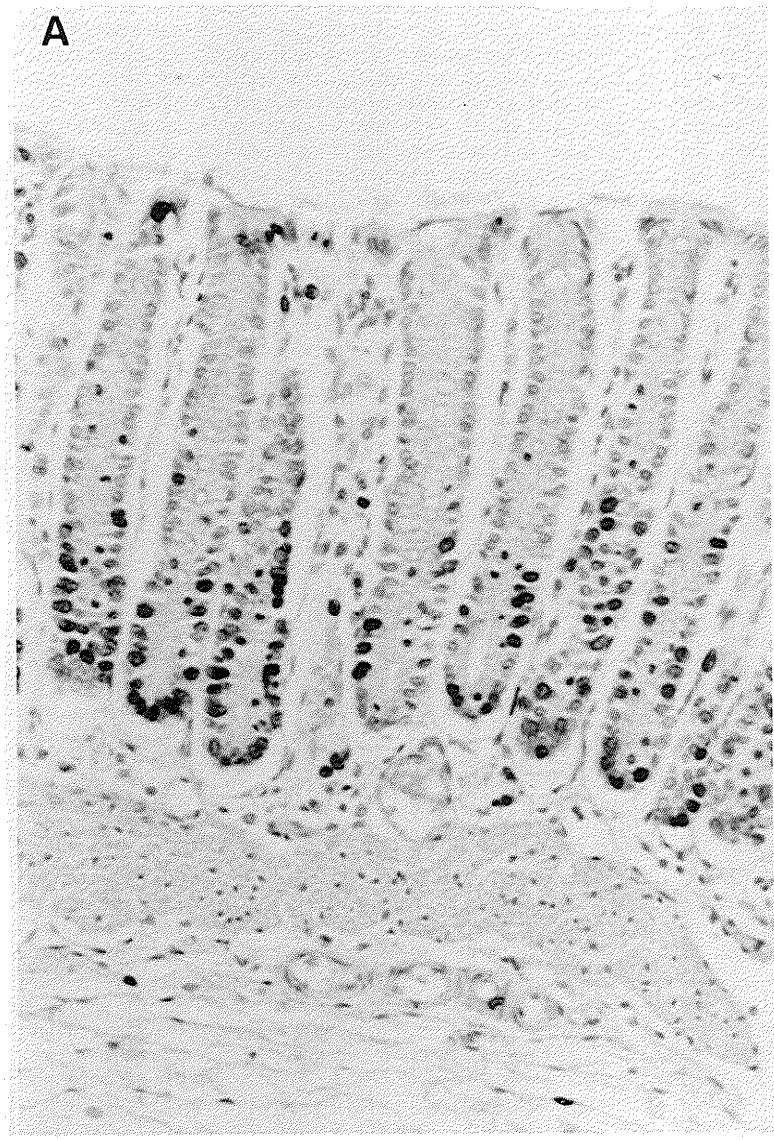
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Table 9. Mitotic cells[1] in the rectal section[2] of colons from rats fed 0.0% or 0.2% CHA for 12 days.

Diet/Time	Crypt Height	# Mitotic Cells	Mitotic Index	Size of Prolif. Zone
CO-3	36.8 ± 2.3^{ab}	2.0 ± 0.3^{b}	2.8 ± 0.4^{b}	9.8 ± 1.3^{bc}
CO-6	34.6 ± 2.5^{b}	1.4 ± 0.2^{b}	2.1 ± 0.4^{b}	6.9 ± 0.4^{c}
CO-9	33.6 ± 1.6^{b}	1.9 ± 0.2^{b}	2.9 ± 0.3^{b}	10.4 ± 0.2^{abc}
CO-12	33.1 ± 1.1^{b}	1.7 ± 0.2^{b}	2.6 ± 0.2^{b}	8.9 ± 0.8^{c}
CHA-3	36.8 ± 0.6^{ab}	2.4 ± 0.2^{b}	3.2 ± 0.2^{ab}	9.7 ± 1.8^{bc}
CHA-6	36.3 ± 1.3^{ab}	2.4 ± 0.7^{b}	3.4 ± 0.9^{ab}	10.6 ± 2.9^{abc}
CHA-9	38.8 ± 0.2^{ab}	4.1 ± 1.2^{a}	5.4 ± 1.6^{a}	15.2 ± 1.9^{a}
CHA-12	40.9 ± 2.7^{a}	3.1 ± 0.2^{ab}	4.0 ± 0.3^{ab}	14.4 ± 1.2^{ab}

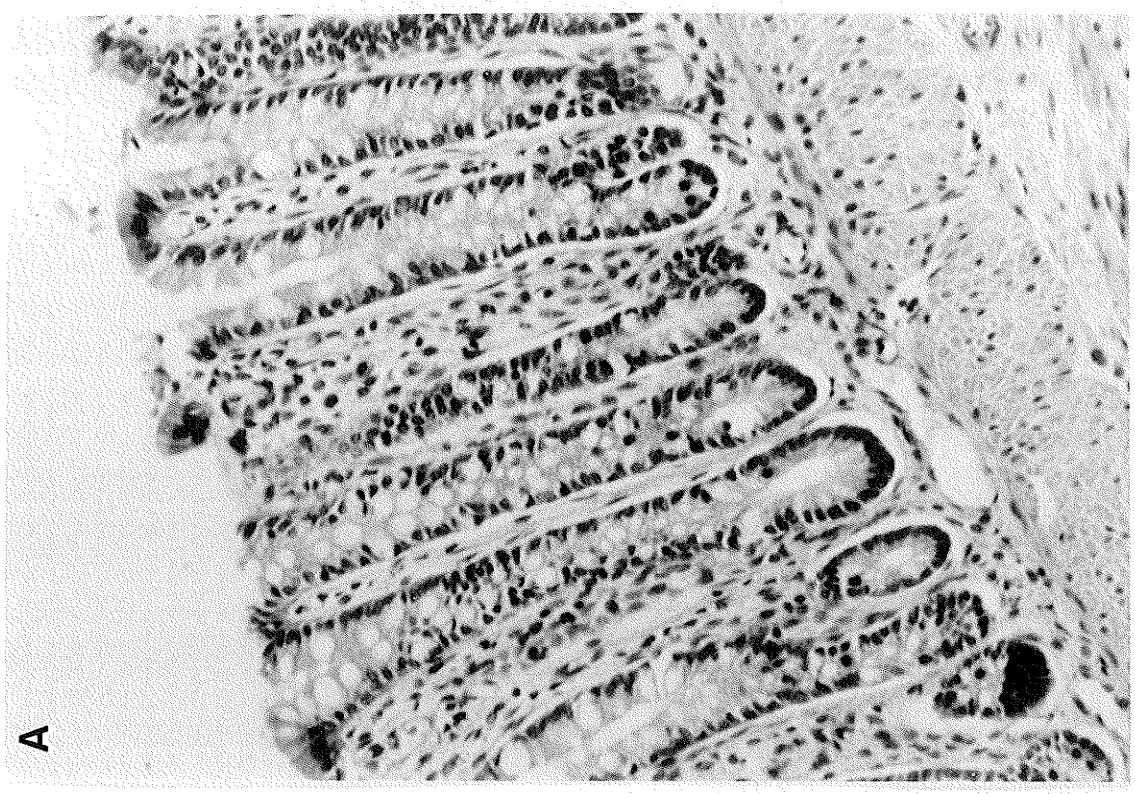
[1]Values expressed as Mean ± SEM. Values within a column with different superscripts differ at $p \leq 0.05$.
[2]cm 1-2 from rectal end of the colon.
CO-3 = 0.0% CHA diet for 3 days etc.
CHA-3 = 0.2% CHA for 3 days etc.

Table 10. Mitotic cells[1] in the mid section[2] of colons from rats fed 0.0% or 0.2% CHA for 12 days.

Diet/Time	Crypt Height	# Mitotic Cells	Mitotic Index	Size of Prolif. Zone
CO-3	36.5 ± 1.6^{c}	2.4 ± 0.6^{b}	3.2 ± 0.8^{b}	11.9 ± 2.3^{ab}
CO-6	36.7 ± 1.2^{c}	1.4 ± 0.2^{b}	2.1 ± 0.3^{b}	10.3 ± 1.2^{b}
CO-9	34.6 ± 1.7^{c}	2.6 ± 0.6^{b}	3.9 ± 1.0^{ab}	11.4 ± 1.3^{b}
CO-12	33.5 ± 1.2^{c}	2.4 ± 0.4^{b}	3.5 ± 0.5^{b}	13.5 ± 1.9^{ab}
CHA-3	37.0 ± 1.4^{bc}	3.0 ± 0.5^{b}	4.4 ± 0.7^{ab}	12.6 ± 0.6^{ab}
CHA-6	39.0 ± 2.5^{abc}	3.3 ± 0.6^{ab}	4.2 ± 0.6^{ab}	14.9 ± 2.3^{ab}
CHA-9	42.1 ± 2.0^{ab}	5.7 ± 2.0^{a}	6.7 ± 2.0^{a}	18.3 ± 3.3^{a}
CHA-12	43.8 ± 1.3^{a}	3.6 ± 0.3^{ab}	4.2 ± 0.4^{ab}	16.2 ± 1.2^{ab}

[1]Values expressed as Mean ± SEM. Values within a column with different superscript differ at $p \leq 0.05$.
[2]cm 5-6 from rectal end.
CO-3 = CO diet for 3 days etc.
CHA-3 = 0.2% CHA for 3 days etc.

Figure 6. Longitudinal section of colonic crypts of rats depicting mitotic cells. (a) Control animal depicting few mitotic cells located in the bottom third of the crypt (40X). (b) Animal fed 0.2% CHA for 9 days. Note increased number of mitotic cells, and increased size of the proliferative zone (40X).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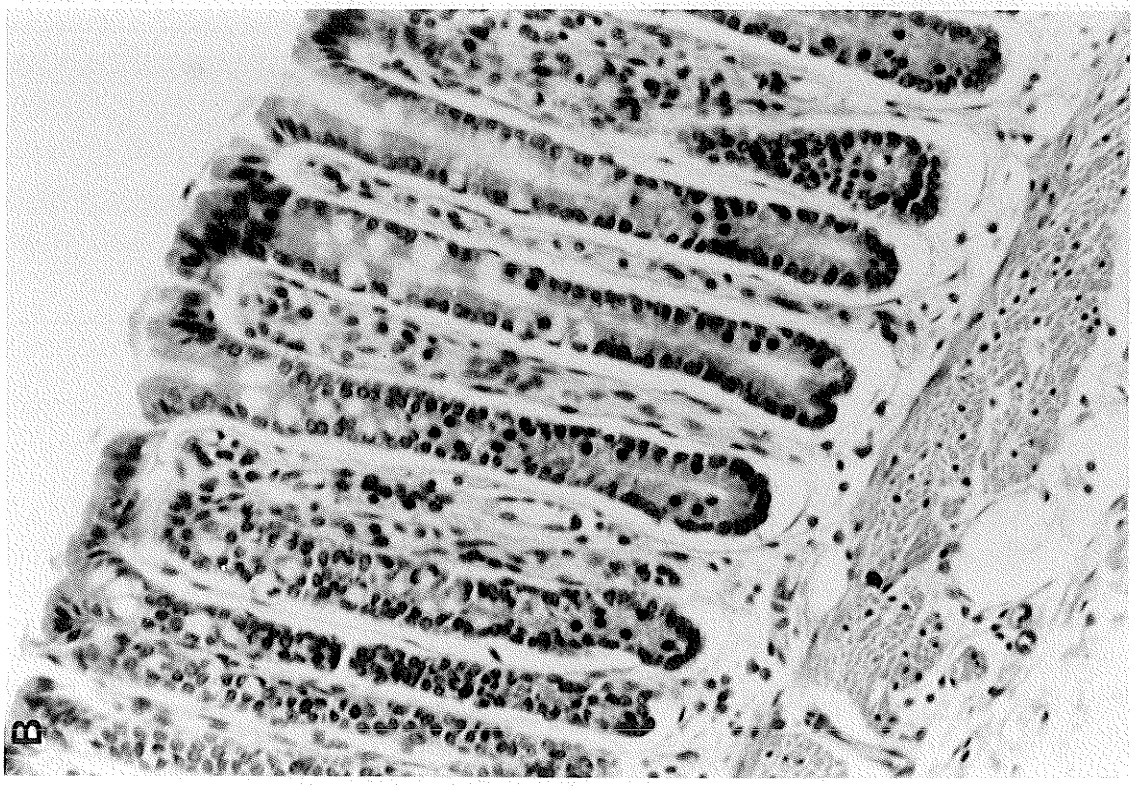
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4. Discussion

The results obtained from both BrUrd labelling activity and metaphase arrest confirm that cholic acid added to the diet increases cell proliferation. It is evident however that some differences exist between the techniques used in measuring proliferation. This confirms previous findings that mitotic and labelling activities do not always correlate and that it is unwise to rely solely on one parameter for indication of cell proliferation (Robblee 1989). Secondly, it is evident that differences exist between rectal and mid regions of the colon. This confirms results from a previous study.

It is interesting to note that several of the proliferative changes occur after 9 days of feeding CHA diet. Prior to this point it appears that the effect of CHA is minimal. This suggests that following the administration of CHA, there is a lag time before an effect is seen. This may represent a defense mechanism where the colon is able to overcome short periods of disturbance, or simply the time it takes for the colon to increase its cell production. It would be interesting to determine if this lag time could be altered by the presence of other compounds such as a carcinogen or calcium.

It appears that many of the changes seen at 9 days subside by 12 days of feeding. This is especially evident for number of BrUrd labelled cells and labelling index. This may represent an initial

response to injury, followed by an adaptation phase where the cells may adjust to the new environment and thus cell proliferation is slowed down. This may suggest that increased cell proliferation is important in the initiation phase of carcinogenesis, but not in later stages of cancer development. Alternatively, this may represent a temporary lull in proliferation that may be overcome with time.

Possibly the most important observation from this study is that CHA induced proliferation is variable. It is evident that the CHA effect is not consistent when different measurements of proliferation are employed. This brings us to question what, if any of these parameters is an effective tool in measuring cancer risk. Proliferation is a non-specific term used to describe any one of or a combination of several parameters. It may be important then to scrutinize the association between different measurements of cell proliferation and neoplastic events in the colon.

The effect of CHA is evident only after a delay period and in some cases subsides shortly thereafter. This may suggest that parameters such a labelling or mitotic index are not a significant marker of risk at later time points, whereas size of proliferative zone or crypt height may be a good indicator of risk at later time points. These findings may allow us to suggest that the appropriateness of measurement depends on the time the measurement is taken. Perhaps not all indicators of increased cell

proliferation are significant at all times.

The varying response of the different proliferative features to a CHA diet at different time points indicates that the physiological response is neither consistent nor clear cut. The results of this study demonstrate that cell proliferation may not be as significant as previously thought.

Chapter VI

VARIABLE EFFECT OF CHOLIC ACID DIET ON THE INDUCTION AND GROWTH OF ACF AND COLONIC TUMORS

1. INTRODUCTION

A diet containing cholic acid (CHA) has been employed to assess the role of bile acids in colon cancer development (Cohen et al., 1980: McSherry et al., 1989). It has been demonstrated that administration of 0.2% (by wt.) CHA in the diet induces a hyperproliferative response in rat and murine colons and enhances tumor incidence in animals initiated with a colon carcinogen (Cohen et al., 1980). It is hypothesized that bile acids mediate their reported tumor promoting activity by acting as mitogens. Therefore, a diet containing CHA is a convenient model which can be meaningfully employed to assess bile acid mediated hyperproliferation, a proposed risk marker of colon carcinogenesis. In a study by Bird (1991), it was observed that animals fed a diet containing CHA had a lower number of aberrant crypt foci (ACF), putative preneoplastic lesions, in their colons. Furthermore, intervention of the disease process with a CHA-containing diet four weeks after initiation with the carcinogen resulted in reduction in number as well as inhibition of the growth of existing ACF. Recently, Magnuson et al.(1993), demonstrated that after 4 weeks of feeding, the total number and average size of ACF were significantly reduced in CHA-fed animals given 1 and 2 injections of azoxymethane (AOM) or methylnitrosourea. These findings were

unexpected and prompted further studies on the effect of CHA on colon carcinogenesis and the mechanism involved in this process.

It was of interest to determine if the CHA-containing diet was acting as a co-carcinogen or tumor promoter. In the literature to date the effect of feeding CHA has been examined only when administered in conjunction with carcinogen exposure. In the present investigation, the effect of a CHA-diet on ACF number and growth and tumor incidence was examined when the CHA-diet was administered immediately following carcinogen injection or after a 1 week delay. It was reasoned that if CHA-diet enhances colon tumor incidence by acting as a promoter, as currently believed, the increased tumor incidence would be noted regardless of time of feeding CHA. Similarly, the growth characteristics of ACF in each group should be similar. Cell proliferation parameters that are associated with CHA ingestion were also measured at a late time point.

2. Materials and Methods

a. Animals

180 male weanling Sprague-Dawley rats were used.

b. Diets

Diets were based on the AIN-76 semi-synthetic diet. Control diet consisted of AIN-76 diet while the cholic acid diet was the AIN-76 diet supplemented with 0.2% cholic acid.

c. Study design

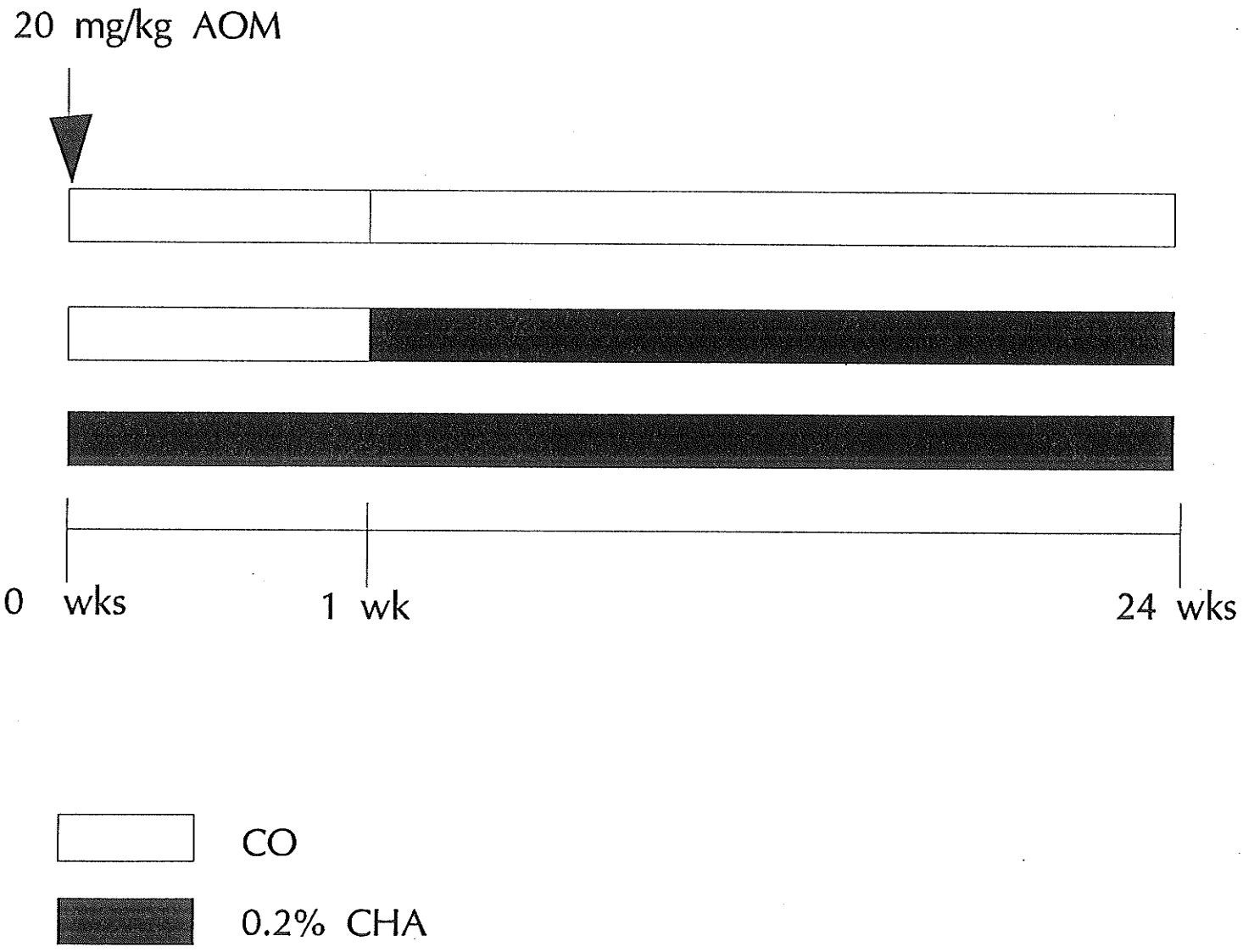
Following carcinogen treatment, animals were randomly assigned to one of the following experimental groups: CO group received control diet throughout the study, CHA group received CHA diet immediately after injection, CHA-delay group received CO diet for one week followed by CHA diet for the remainder of the study (24 weeks, Fig. 8). Animals were allowed to eat the designated diet ad libitum throughout the study. Initial body weights were recorded, and subsequent weighing took place at three week intervals.

At three week intervals three animals per group were terminated by carbon dioxide asphyxiation. Colons were removed, fixed in 4% paraformaldehyde, and prepared for enumeration of ACF as previously described (Chapter 3). The number, size, distribution, and crypt multiplicity of ACF were determined for the entire length of the colon.

Proliferative status of the colonic epithelium was assessed by three different procedures. At 24 weeks, four animals per group were injected with bromodeoxyuridine (BrUrd 50 mg/kg body weight, Sigma Chemical Co.), one hour prior to termination for determination of BrUrd labelled cells. In order to measure mitotic activity, four animals per group were injected with colchicine (1mg/kg body weight Sigma Chemical co.), two hour prior to termination. The number of cells exhibiting proliferating cell nuclear antigen (PCNA) was also assessed. (See Appendix B for detailed description of immunohistochemical procedures).

At 24 weeks, all remaining animals were terminated as faecal blood was evident. Animals were coded to conceal the identity of treatment group until histopathological evaluation was completed. The location, appearance, and dimensions of all suspicious macroscopic lesions were recorded. Each lesion was excised and fixed in 4% paraformaldehyde or 70% ethanol. Lesions were embedded in paraffin, sectioned at 5 microns 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. Histopathological assessments of tumors was carried out by I. Carr, Department of Pathology, University of Manitoba.

Figure 7. Schematic representation of experimental protocol for rats given a diet containing 0.2% CHA beginning at different time periods.



3. Results

Diet had no consistent effect on body weight at various time points (Table 11). Throughout the study, the cholic acid fed animals maintained slightly lower body weights.

The effect of the various treatment protocols on the total number of ACF is shown in Figure 9. Corn oil fed animals had consistently more ACF per colon at all time points. The differences were significant at all time points except week 12 and 15. There was no significant difference between the CHA and CHA-delay groups. In all groups, a period was noted which was accompanied by a decline in the number of ACF followed by an increase. The time and degree by which this occurred varied among the groups. For instance, in the CO group the decline was seen by week 12, whereas both CHA groups showed a decline by weeks 15 to 18.

Mean aberrant crypts per focus (AC/focus) is shown in Figure 10. By week 15 the effect of varying CHA treatment was apparent. The CHA diet had the highest mean (2.7±0.2) and corn oil diet had the lowest mean (2.3±0.3) whereas the CHA-delay group exhibited an intermediate value (2.6±0.1). The trend established here remained consistent throughout the study. By week 21 the values for the CHA groups were significantly higher than for the CO group. CO diet had consistently more ACF consisting of 1-4 crypts per focus (Table 12). This effect was significant at most time points studied. The percent of total ACF consisting of 5 or more crypts per focus is

Table 11. Body weights of animals receiving CO, CHA, or CHA-delay diet.

	Treatment Groups		
WEEK	CO[1]	CHA[1]	CHA-delay[1]
0	111.6 ± 1.1[ab]	114.1 ± 1.0[a]	110.7 ± 1.1[b]
3	265.6 ± 2.6[ab]	273.6 ± 4.6[a]	259.3 ± 2.8[b]
6	379.0 ± 3.9[a]	372.7 ± 3.9[ab]	365.5 ± 3.9[b]
9	456.3 ± 4.7[a]	448.3 ± 4.9[ab]	437.3 ± 5.0[b]
12	506.5 ± 5.8[a]	502.1 ± 5.9[a]	482.4 ± 5.4[b]
15	546.5 ± 5.9[a]	545.6 ± 6.5[a]	524.4 ± 6.5[b]
18	571.7 ± 6.3[a]	572.3 ± 7.0[a]	552.2 ± 6.2[b]
21	597.5 ± 8.1[a]	594.5 ± 8.0[ab]	574.0 ± 6.8[b]
24	625.3 ± 8.8[a]	611.3 ± 9.3[a]	600.4 ± 8.2[a]

[1] Grams; mean ± SEM.
[ab] Means within a row with different superscripts differ at $p \leq 0.05$.

Figure 8. Effect of CO. CHA, or CHA-delay diet on total number of ACF

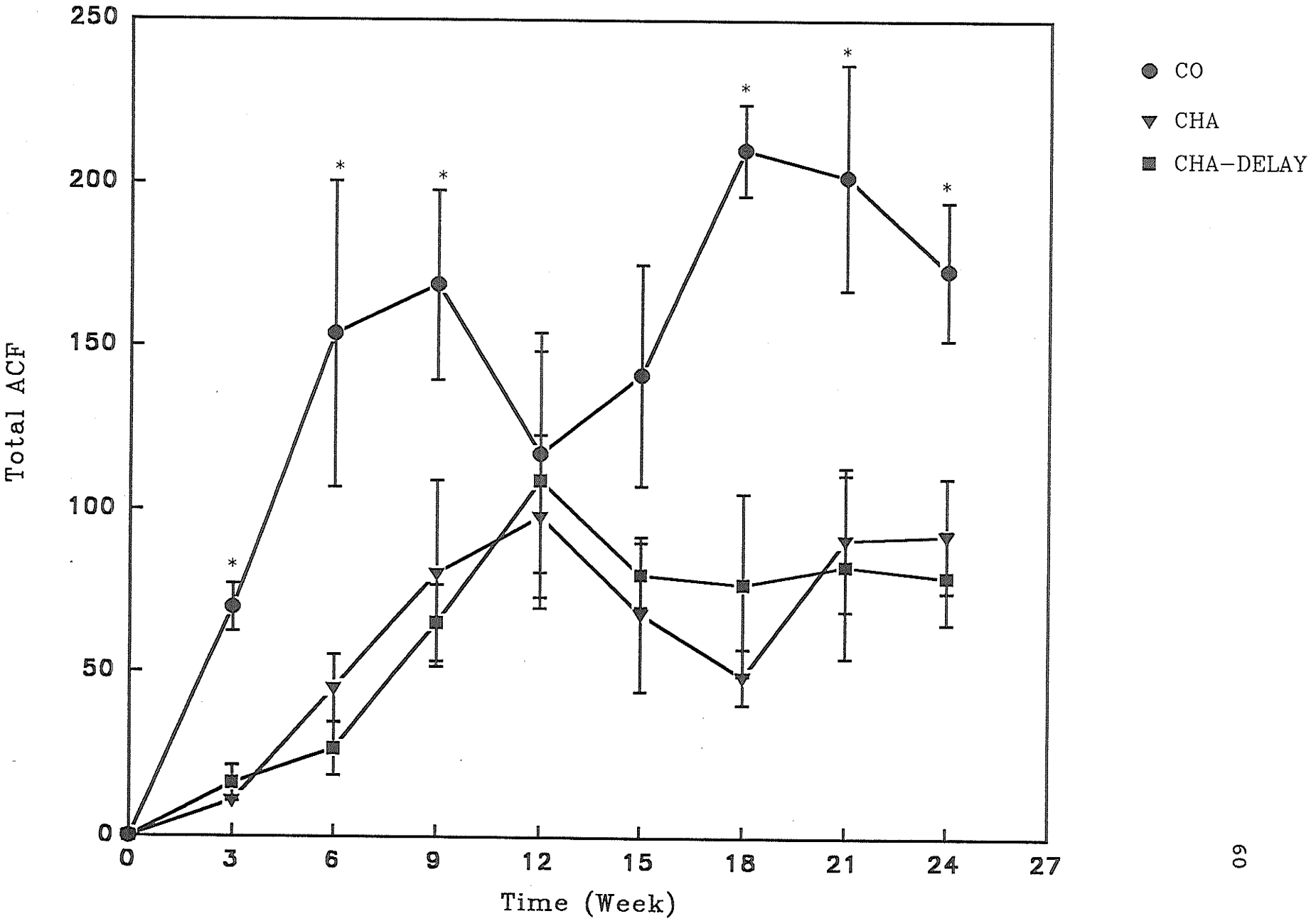


Figure 9. Mean AC/focus for animals consuming CO, CHA, or CHA–delay diets

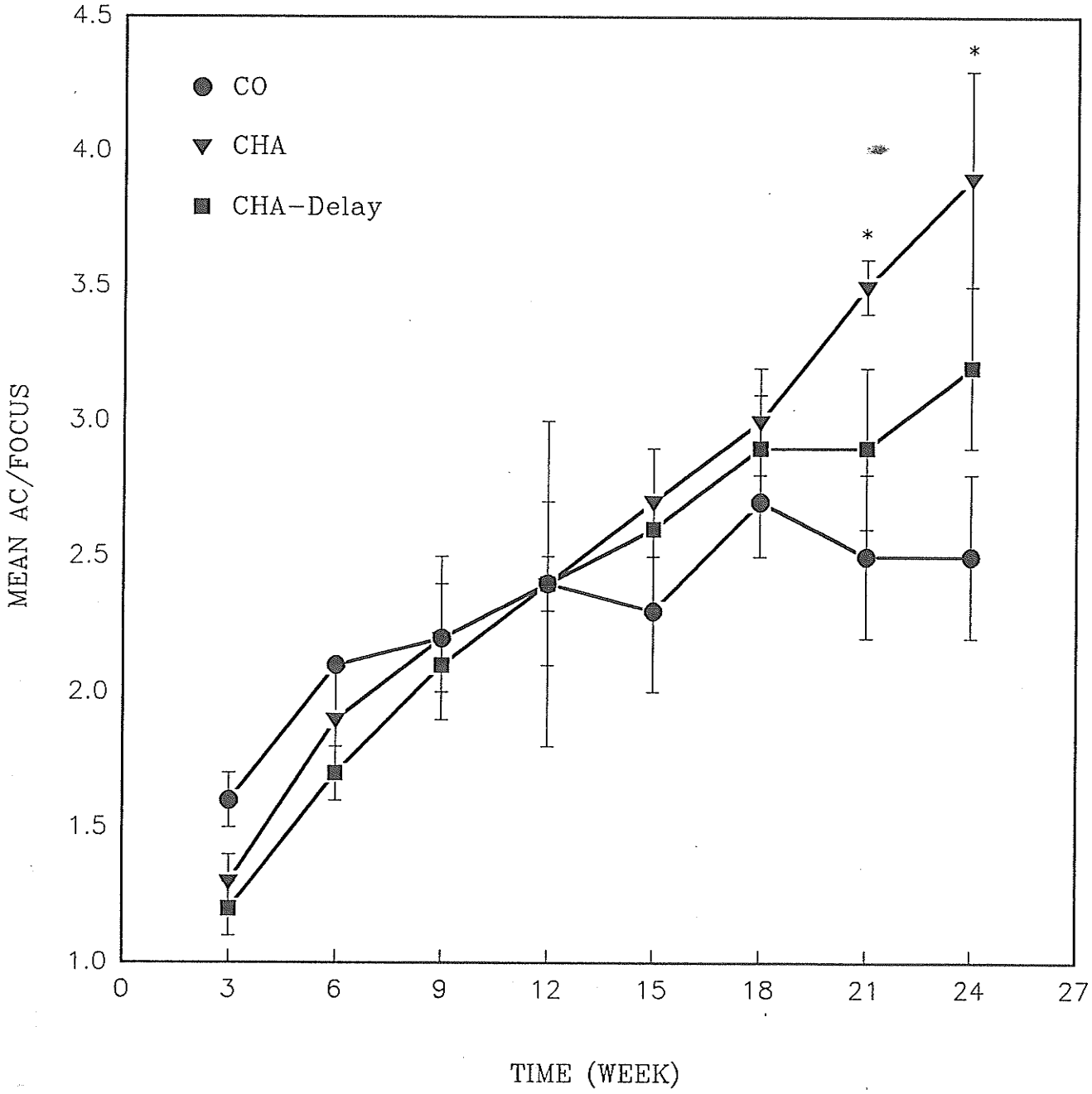


Table 12. Population of ACF consisting of different multiplicities for animals consuming CO, CHA, or CHA-delay diets.

WEEK	CRYPTS/ FOCUS	CO[1]	CHA[1]	CHA-DELAY[1]
3	1-4	69.7 ± 7.3[a]	11.0 ± 2.3[b]	16.3 ± 5.4[b]
	5-8	0.0	0.0	0.0
	≥9	0.0	0.0	0.0
6	1-4	150.3 ± 46.2[a]	44.0 ± 9.6[b]	26.7 ± 8.0[b]
	5-8	3.3 ± 0.7[a]	0.7 ± 0.3[b]	0.0[b]
	≥9	0.0	0.0	0.0
9	1-4	162 ± 30.9[a]	75.7 ± 27.0[ab]	71.3 ± 15.6[b]
	5-8	6.3 ± 2.3[a]	4.7 ± 3.2[a]	1.3 ± 0.9[a]
	≥9	0.0	0.0	0.0
12	1-4	112.7 ± 35.8[a]	82.3 ± 22.3[a]	106.0 ± 40.0[a]
	5-8	4.3 ± 2.4[a]	14.0 ± 5.5[a]	2.7 ± 0.7[a]
	≥9	0.3 ± 0.3[a]	1.7 ± 0.9[a]	0.3 ± 0.3[a]
15	1-4	132.3 ± 36.1[a]	57.7 ± 17.6[a]	73.3 ± 9.2[a]
	5-8	8.7 ± 2.0[a]	9.7 ± 6.2[a]	6.7 ± 0.9[a]
	≥9	0.3 ± 0.3[a]	0.7 ± 0.3[a]	0.0[a]
18	1-4	188.0 ± 7.0[a]	42.3 ± 9.9[b]	66.0 ± 24.1[b]
	5-8	21.3 ± 6.7[a]	5.7 ± 2.4[a]	10.0 ± 3.1[a]
	≥9	1.0 ± 0.6[a]	0.7 ± 0.3[a]	1.0 ± 1.0[a]
21	1-4	182.3 ± 31.8[a]	70.7 ± 15.3[b]	69.0 ± 18.2[b]
	5-8	17.7 ± 5.4[a]	19.3 ± 5.2[a]	12.0 ± 9.5[a]
	≥9	2.0 ± 1.2[a]	1.7 ± 1.2[a]	1.7 ± 0.7[a]
24	1-4	157.8 ± 17.7[a]	60.8 ± 6.5[b]	65.0 ± 11.1[b]
	5-8	21.6 ± 7.1[a]	16.8 ± 3.9[a]	11.4 ± 1.9[a]
	≥9	1.8 ± 0.9[a]	5.0 ± 2.4[a]	1.8 ± 0.9[a]

1 Mean ± SEM.
[ab] Means within a row with different subscripts differ at $p \leq 0.05$.

depicted in Figure 11. The CHA group generally had the highest value for ACF with ≥5 or ≥9 crypts followed by CHA-delay and then CO. Size of ACF was recorded and is reported in Appendix D-2. Although CHA diet seems to exert a significant reducing effect at early time points (weeks 3-9), this trend seems to diminish with time.

Tumor incidence data is presented in Table 13. Total number of tumors and total number of animals with tumors is significantly higher in CHA fed animals than CO fed animals (15 vs. 5) respectively ($p \leq 0.05$). However, the tumor incidence of the CHA-delay group was not significantly different from the CO group.

Quantification of the proliferative activity of the colons by metaphase arrest technique is shown in Table 14. The groups fed CHA generally had higher crypt height compared to the control group. This effect is most pronounced in the mid region of the colon for the CHA group ($p \leq 0.05$). The CHA-delay group exhibited an intermediate value in this regard. In the rectal region both CHA groups had increased size of proliferative zone. Enumeration of the BrUrd labelled cells, representing cells engaged in DNA synthesis revealed differences in crypt height (rectal and mid region) and size of proliferative zone (mid region) for the CHA group (Table 15). PCNA labelling indices were similar among all groups with the exception of crypt height which was higher for the CHA group (Appendix D-2).

Figure 10. Percent of ACF consisting of 5–8 or ≥9 crypts/focus

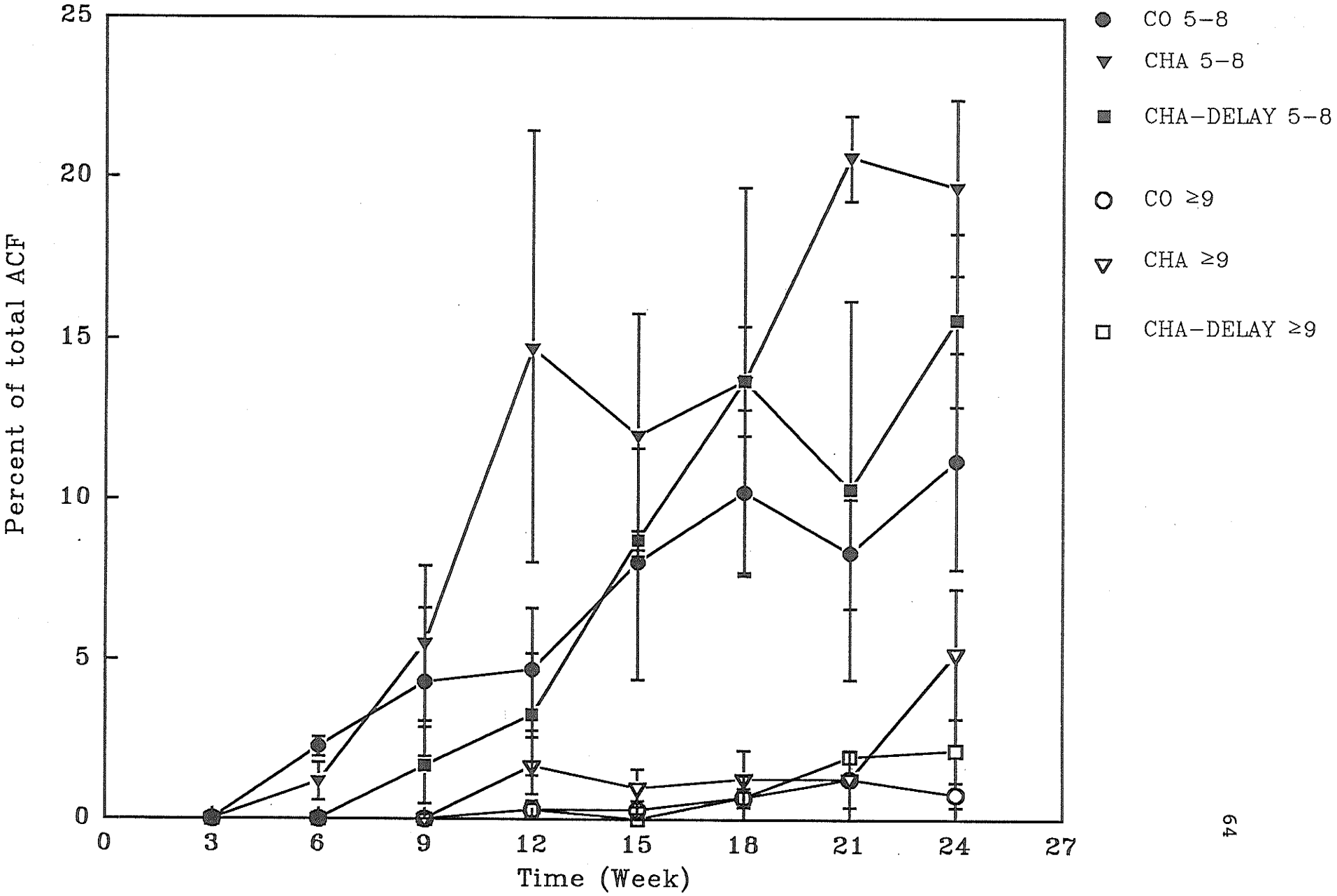


Table 13 Tumor pathology from the colons of rats consuming CO, CHA, or CHA-delay diets.

	Diet		
	CO	CHA	CHA-delay
N	38	39	39
Rats With tumors	5 (13.2%)[a]	15 (39.5%)[b]	9 (23.1%)[a]
Tumors/Tumor Bearing Animal	1.20	1.46	1.56
Rats with Adenomas	2 (5.3%)	8 (20.5%)	5 (12.8%)
Rats with Adenocarcinomas	4 (10.5%)	10 (25.6%)	6 (15.4%)

[ab] Values within a row with different superscripts differ at $p \leq 0.05$.

Table 14 Effect of CO, CHA, or CHA-delay diet on the mitotic activity of colonic epithelium at week 24[1]

Rectal region[2]

GROUP	CRYPT HEIGHT	# MITOTIC FIGURES	MITOTIC INDEX	SIZE OF PROLIF. ZONE
CO	32.5 ± 2.3[a]	2.6 ± 0.3[a]	3.9 ± 0.3[a]	11.5 ± 1.3[b]
CHA	38.5 ± 2.4[a]	3.2 ± 0.4[a]	4.2 ± 0.4[a]	15.5 ± 1.2[a]
CHA-DELAY	37.3 ± 2.1[a]	3.6 ± 0.3[a]	4.8 ± 0.3[a]	15.0 ± 0.7[a]

Mid section[3]

CO	31.5 ± 2.6[b]	2.6 ± 0.2[a]	4.1 ± 0.2[a]	11.5 ± 1.6[a]
CHA	41.5 ± 1.4[a]	4.0 ± 0.4[a]	4.8 ± 0.3[a]	16.5 ± 1.3[a]
CHA-DELAY	36.3 ± 2.4[ab]	4.2 ± 1.2[a]	5.8 ± 1.7[a]	16.3 ± 2.9[a]

[1]mean ± SEM. 4 animals/group.
[2]cm 1-2 from rectal end of the colon.
[3]cm 7-8 from rectal end of the colon.
[ab]Means within a column in each region with different superscripts differ at $p \leq 0.05$.

Table 15 Effect of CO, CHA, or CHA-delay diet on the number of BrUrd labelled cells in the colonic epithelium[1].

Rectal end[2]

GROUP	CRYPT HEIGHT	# LABELLED CELLS	LABELLING INDEX	SIZE OF PROLIF. ZONE
CO	37.4 ± 1.2^{ab}	4.7 ± 0.39^{a}	6.2 ± 0.5^{a}	13.6 ± 1.6^{a}
CHA	39.4 ± 1.1^{a}	4.6 ± 1.2^{a}	5.9 ± 1.5^{a}	13.8 ± 1.1^{a}
CHA-DELAY	34.4 ± 1.2^{b}	3.9 ± 0.5^{a}	5.7 ± 0.8^{a}	11.7 ± 0.8^{a}

Mid section[3]

CO	32.4 ± 1.8^{b}	3.6 ± 0.4^{a}	5.6 ± 0.6^{a}	11.1 ± 0.4^{b}
CHA	44.1 ± 2.1^{a}	7.3 ± 1.8^{a}	8.4 ± 2.0^{a}	16.8 ± 1.4^{a}
CHA-DELAY	37.7 ± 1.4^{b}	4.5 ± 0.9^{a}	5.9 ± 1.1^{a}	13.9 ± 1.2^{b}

[1] Mean ± SEM. 4 animals/group.
[2]cm 1-2 from the rectal end of the colon.
[3]cm 7-8 from the rectal end of the colon.
[ab]Means within a column with different superscripts differ at $p \leq 0.05$

4. Discussion

The principal objective of the present investigation was to assess the effect of CHA-diet employing two different experimental protocols on the induction and growth characteristics of ACF as well as on colon tumor development. The main difference between the two groups treated with CHA was that one group began the CHA-diet on the day of carcinogen treatment whereas the second group began the CHA-diet one week after the carcinogen treatment.

The main findings of this investigation are that the CHA and CHA-delay groups behaved quite differently from each other when compared to the control group with respect to growth characteristics of ACF as well as tumor incidence.

The enumeration of the number and crypt multiplicity of ACF was carried out as end points to assess the preneoplastic changes occurring in the three groups. Previously it has been demonstrated that crypt multiplicity was an important indicator of cancer development and tumor incidence (Bird et al., 1989; Tudek et al., 1989). In a recent study, (Magnuson, unpublished results) where CHA was fed in conjunction with the carcinogen it was demonstrated that in spite of a marked decrease in number of ACF, the number of ACF with higher multiplicity was increased. Findings of the present study supports previous reports that ACF with higher multiplicity represent advanced lesions and their number and growth must be taken into account while predicting tumor outcome. In this

study it was evident that after a time lag of approximately 18 weeks, the CHA group began to exhibit a relatively higher proportion of ACF with advanced growth features, the CHA-delay group exhibited intermediate values, and the control group had the least proportion of ACF with advanced features. This trend was mimicked in the tumor incidence data.

The observation that during the development of the disease, some ACF may go through a remodelling phase as evident by a sharp decrease in total number of ACF. This observation is interesting and has been documented previously (McLellan et al, 1991b). Similar observations have been made in other organs demonstrating regression or reversion of preneoplastic lesions to normal phenotype (Farber and Rubin, 1991).

Differences among CHA, CHA-delay, and control groups with respect to ACF parameters were most evident at later time points. It is reasonable to suggest that the main effect of CHA-diet was exerted at a very early time point, likely within the first one or two weeks after the carcinogen injection, otherwise CHA and CHA-delay groups should have behaved in a similar manner. It is probable that when CHA-diet is administered in conjunction with carcinogen administration, it may act as a co-carcinogen either by increasing the potency of AOM and/or by increasing the number of initiated cells with higher propensity to develop into cancer.

It is not clear from the tumor incidence reported in this study that CHA is acting as a tumor promoter. Cruse and co-workers (1981) found no evidence of promotion by intragastric instillations of CHA in carcinogen initiated rats. The tumor promoting effect has been demonstrated in studies where CHA is fed or administered intrarectally to initiated rats (Reddy et al., 1976; Reddy et al., 1977). The findings of the present study convincingly demonstrate that the ACF-reducing effect of CHA-diet is independent of its ability to enhance colon carcinogenesis. For instance, CHA group and CHA-delay group both had lower number of ACF, however, tumor enhancing effect was noted only in the CHA group. It appears that the protocol employed is central to the tumor enhancing effects of CHA diet. This suggests that the period immediately after initiation appears to be critical and that CHA may be acting as a co-carcinogen rather than a tumor promoter. It would be interesting to determine if a further delay in the intervention with CHA diet (2-4 weeks) would completely abolish the tumor enhancing effect of this diet.

It is suggested that increased cell proliferation is the mechanism responsible for CHA's promotional effect (Cohen et al., 1980; Deschner et al., 1981). In this study, only crypt height and size of proliferative zone were increased when proliferative indices were assessed at a later time point. This observation is perplexing in view of the reported mitogenic effect of CHA containing diet on rodent colons at early time points. It is

possible that even normal appearing crypts go through some adaptive process to overcome the toxic effect of elevated levels of bile acids in the colonic epithelium.

In summary, it was demonstrated that feeding a 0.2% CHA diet to carcinogen initiated rats reduces the number of ACF in the colonic epithelium. The time of introduction of CHA appears significant in determining the growth characteristics of ACF and tumor incidence. These results emphasize the need to scrutinize experimental protocols in the identification of potential tumor promoters or inhibitors.

Chapter VII

EFFECT OF LATE TIME ADMINISTRATION OF CHOLIC ACID DIET ON NUMBER, MULTIPLICITY, AND SIZE OF ACF

1. Introduction

In the previous study (chapter VI) it was demonstrated that a diet containing CHA was quite effective in reducing the number of ACF. In addition it was evident that this effect was unrelated to the tumor enhancing effect of a diet containing CHA. In view of these novel findings, it was of interest to determine if intervention with CHA at late time points has any effect on the number and multiplicity of ACF. It was reasoned that once lesions were initiated and allowed to develop for 21 weeks they may be more stable and respond differently to the number and growth modulating effect of CHA-diet. However, if indeed CHA-diet eliminates ACF by exerting a cytotoxic response and/or by reverting the ACF to normal phenotype, then it was presumed that ACF with different growth characteristics may respond differently to the CHA-diet. In view of these considerations, the main objective of this investigation was to determine:

1. if the CHA-diet will affect the number and growth of ACF when administered several weeks after carcinogen administration

2. if the CHA-diet will affect ACF differently depending on their growth features.

2. Materials and Methods

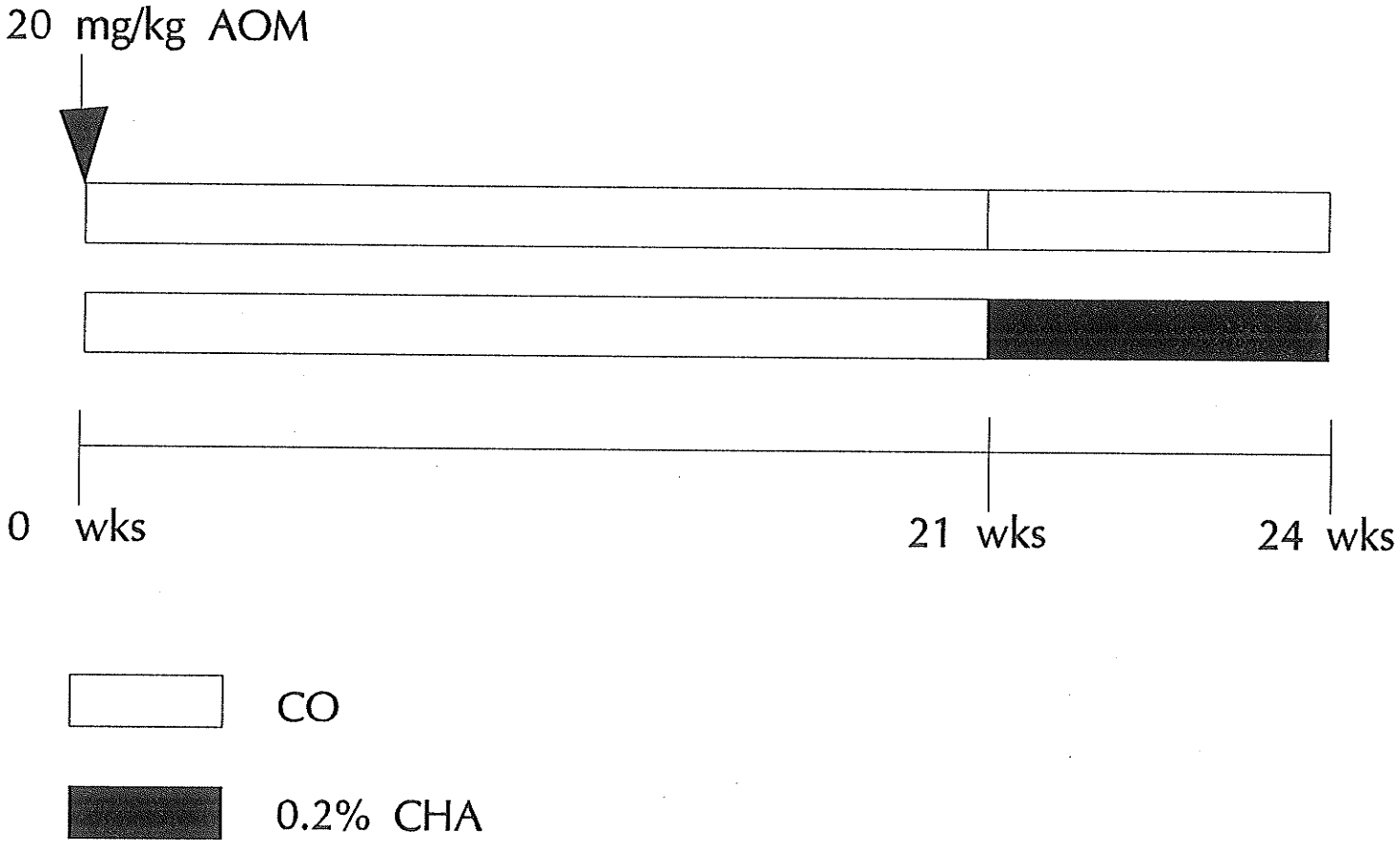
a. Animals

Male weanling Sprague-Dawley rats were used.

b. Study design

Schematic representation of experimental protocol is presented in Figure 12. Following acclimatization, all animals received a single injection of azoxymethane (AOM) (20mg/kg) and were maintained on CO diet for 21 weeks. At this time, 3 animals were sacrificed and colons were removed and evaluated for ACF (CO-1 group). The remaining animals were divided into 2 groups. Five animals were maintained on CO diet (CO-2 group), while 6 animals were switched to 0.2% CHA diet (CHA group). After 3 weeks on their respective diets, all animals were terminated and colons were removed and evaluated for number, size and multiplicity of ACF.

Figure 11. Schematic representation of experimental protocol for rats given CO diet for 21 weeks followed by three weeks of CHA-diet.



3. Results

Administration of CHA diet for 3 weeks following 21 week of CO diet significantly reduced total number of ACF from 202 to 70 (Figure 13).

Crypt multiplicity for the three groups is shown in Table 16. CHA diet significantly reduced the number of ACF consisting of 1-4 crypts/focus, while ACF with higher crypt multiplicity were not significantly altered (Table 16 and Figure 14).

Growth features of ACF with 1-4 crypt multiplicity were scrutinized. It was apparent that although ACF had similar crypt multiplicity they varied a great deal in size. This suggested that some ACF consisted of large, dilated crypts whereas others consisted of smaller more constricted crypts. It was noted that ACF with 1-2 crypts were more vulnerable to elimination by CHA diet regardless of their size. This trend was not seen among the ACF with 3-4 crypt multiplicity.

The CHA diet eliminated or reduced the number of ACF with 1-2 crypts/focus in all size categories (Table 17). This trend was not evident among ACF with 3-4 crypts/focus as significant reduction was evident in only the ACF with larger size (5-10 x $10^{-2}mm^2$).

Figure 12. Reduction of total number of ACF by three weeks of CHA–diet following 21 weeks of CO diet

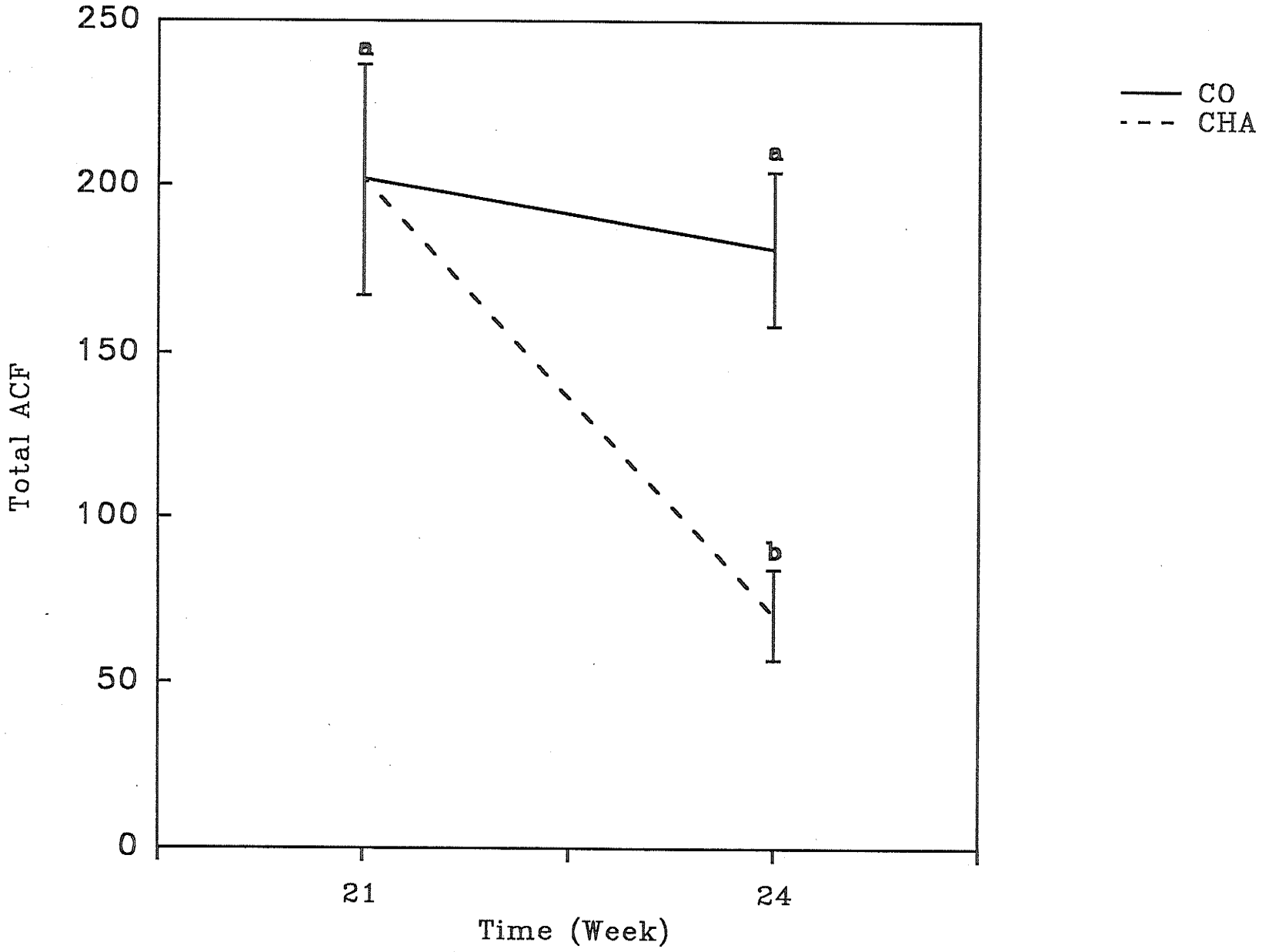


Table 16. Multiplicity of ACF[1] from animals fed CO-diet for 21 and 24 weeks, or CO-diet for 21 weeks followed by CHA-diet for three weeks.

CRYPTS/FOCUS	CO-1[2]	CO-2[3]	CHA[4]
1-2	118.3 ± 22.2[a]	108.4 ± 11.0[a]	33.0 ± 5.0[b]
3-4	64.0 ± 12.6[a]	49.4 ± 8.6[ab]	25.7 ± 6.2[b]
5-6	13.0 ± 4.5[a]	17.0 ± 6.0[a]	8.3 ± 2.8[a]
7-8	4.7 ± 1.3[a]	4.6 ± 1.1[a]	2.0 ± 1.2[a]
9-10	1.3 ± 0.9[a]	1.0 ± 0.5[a]	1.3 ± 0.6[a]
>10	0.7 ± 0.3[a]	0.8 ± 0.6[a]	0.2 ± 0.2[a]

[1]Mean ± SEM.
[2]Animals fed CO diet for 21 weeks.
[3]Animals fed CO diet for 24 weeks.
[4]Animals fed CO diet for 21 weeks followed by 0.2% CHA diet for 3 weeks.
[ab]Means within a row with different superscripts differ at $p<0.05$.

Figure 13. Graphic depiction of the reduction of ACF consisting of 1–2 and 5–6 crypts/focus by three weeks of feeding CHA–diet

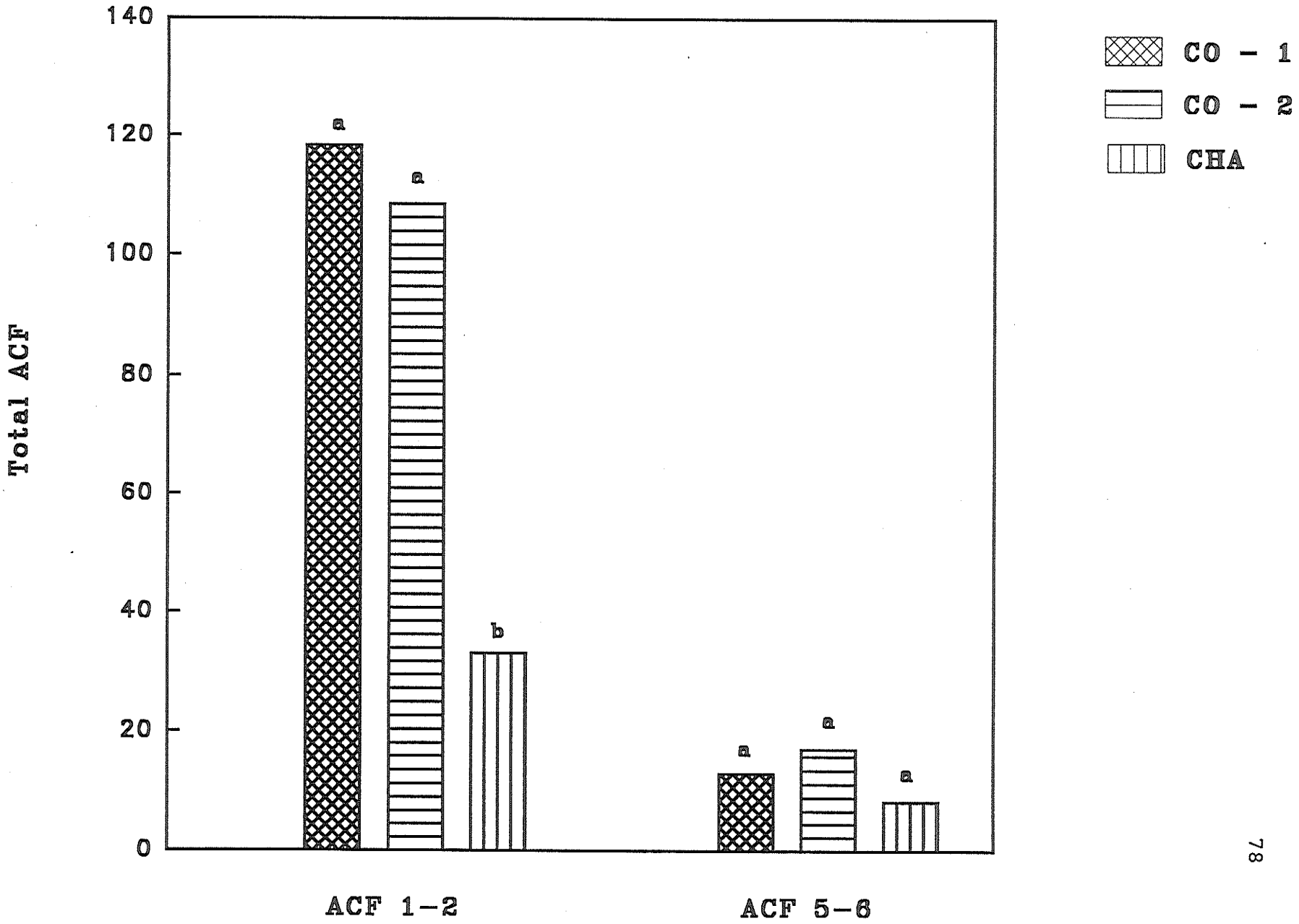


Table 17. Size of ACF[1] in colons of rats consuming CO diet for 21 weeks followed by CHA-diet for three weeks.

ACF consisting of 1-2 crypts/focus

SIZE $X10^{-2}mm^2$	CO-1[2]	CO-2[3]	CHA[4]
1-2	44.3 ± 9.0[a]	47.2 ± 3.8[a]	13.7 ± 2.8[b]
3-4	49.0 ± 11.7[a]	40.4 ± 5.9[a]	14.8 ± 1.9[b]
5-6	19.0 ± 2.6[a]	17.8 ± 3.2[a]	4.0 ± 0.7[b]

ACF consisting of 3-4 crypts/focus

SIZE $X10^{-2}mm^2$	CO-1[2]	CO-2[3]	CHA[4]
1-2	0.0 ± 0.0[a]	0.4 ± 0.2[a]	0.0 ± 0.0[a]
3-4	12.3 ± 2.3[a]	14.2 ± 3.6[a]	9.5 ± 2.9[a]
5-6	26.7 ± 7.9[a]	16.4 ± 3.9[ab]	8.5 ± 2.4[b]
7-8	10.7 ± 2.7[a]	7.2 ± 1.3[ab]	4.5 ± 1.3[b]
9-10	10.0 ± 2.0[a]	6.8 ± 1.1[a]	2.7 ± 0.7[b]

[1]Values expressed as mean ± SEM. Values within a row with different superscripts differ at $p \leq 0.05$.
[2]Animals fed CO diet for 21 weeks.
[3]Animals fed CO diet for 24 weeks.
[4]Animals fed CO diet for 21 weeks followed by 0.2% CHA diet for 3 weeks.

4. Discussion

The main finding of this study are that feeding a diet containing 0.2% CHA at a late time point significantly reduced the number of ACF in the colons of rats initiated with a colon carcinogen. The CHA intervention significantly reduced those ACF with fewer (1-4) crypts per focus. Growth features of the ACF were assessed by measuring size (area) and crypt multiplicity (crypts/focus). Previously we have reported that CHA treatment not only reduced the number of ACF but also significantly reduced the average size of each ACF (Bird, 1991). Interestingly average crypt multiplicity (mean AC/focus) did not change among the groups fed CHA-diet when compared to corn oil fed animals (Bird, 1991). This observation suggested that ACF with large area, thus consisting of more dilated crypts are more susceptible to elimination by CHA diet. Findings of this study support this concept when ACF with 3-4 crypt multiplicity were considered but not those ACF with 1-2 crypt multiplicity.

By employing CHA diet, it was demonstrated that ACF with higher crypt multiplicity represent biologically different lesions than those with lower crypt multiplicity. Based on the findings of the present study, it is also proposed that biological heterogeneity may exist among ACF with similar crypt multiplicity. The cellular and molecular basis for these observations remains elusive.

Chapter VIII

GENERAL DISCUSSION

A. Cholic acid and colon carcinogenesis

This research employed aberrant crypt foci (ACF) as the biological endpoint to test the hypothesis that CHA-diet exerts a modulating effect in the development of colon cancer. This hypothesis was based on the contention that increased levels of bile acids in the colonic lumen have been associated with increased cancer risk, and that a CHA-diet has increased colonic tumor incidence in rodents (Cohen et al., 1980: McSherry et al., 1989). Paradoxically, it has also been demonstrated that CHA-diet reduces the number of ACF in the rodent colon (Bird, 1991; Magnuson and Bird, 1993). Based on this information it was of interest to determine the role of experimental protocol on ACF characteristics and tumor incidence when the CHA diet was employed. It was also of interest to measure cell proliferation to determine if it contributed to reduction of ACF, as CHA-diet is thought to induce a mitogenic response.

Although the effect of bile acids on colon cancer development has been extensively studied, little information exists on the effect of low levels of bile acid on colonic epithelium. Short term feeding of diets containing 0.025%, 0.05%, or 0.1% CHA resulted in variable increases of cell proliferation. The differences were not consistent between different types of measurement, nor at different locations in the colon.

When 0.2% CHA was administered and cell proliferation measurements were taken at three day intervals over a 12 day period, results again differed, however there was a general increase in number of cells in S-phase. This further supports the contention that cell proliferation measurements are not a consistent marker and should not be relied on as a single means of determining cancer risk. It is also interesting to note that many of the proliferative differences began to disappear by day 12. This may reflect an adaptive response that takes place in the colon, further questioning the validity of proliferation parameters. Information from the two preliminary studies suggest that enhanced cell proliferation is not necessarily the main mechanism by which CHA exerts its effect.

The variable effect of CHA-diet on the number and growth of ACF and final tumor outcome was the most important information generated in this dissertation. Monitoring the number, multiplicity, and size of ACF provided valuable information about these precursor lesions. It was evident that total number of ACF did not reflect final tumor outcome, thus it is unlikely that absolute number of ACF is a reliable predictor of tumor incidence. Size is not likely a good predictor of tumor outcome as polyps and tumors consist of many crypts of various sizes, and not a single large crypt. Information generated from this study demonstrated that CHA diet reduced the ACF consisting of fewer crypts/focus, while foci with higher multiplicity may have continued to develop into polyps or adenomas.

A second major finding of this study is that CHA did not increase final tumor incidence as markedly when its implementation was delayed for one week following carcinogen administration as when its implementation is immediately after carcinogen injection. This suggests that CHA may not be acting as a promoter, but rather a co-carcinogen. This information may have far reaching implications that may be applied to other organ systems using other test agents.

The final study in this series indicated that CHA can exert its ACF reducing effect when implemented at late time points and demonstrated that ACF with higher crypt multiplicity (>5) resisted the effect of CHA. This indicates that ACF exist in a dynamic state and their remodelling is a continuous process. Furthermore the ACF model was used to determine if those ACF that were eliminated or remodeled by CHA diet possessed particular morphological features.

From these results it can be suggested that CHA in the diet removes superficial lesions, leaving behind and possibly enhancing the growth of lesions that will go on to form polyps or adenomas. If this is indeed the case, it is possible that CHA-diet can be used in conjunction with ACF as a model that allows for the study of the stepwise development of colon carcinogenesis.

B. Major Findings and Implications

The findings of this dissertation have provided fundamental

information on the feasibility of using CHA-diet in conjunction with ACF in the rodent as a means to study the stepwise development of colon cancer. Of primary importance, this research has established that a modulator of colon carcinogenesis may exert different effects on tumor outcome depending on the experimental protocol employed. Secondly it was demonstrated that total number of ACF may not predict disease outcome. Consequently a detailed analysis of the growth characteristics of ACF is essential in order to assess disease risk. Finally this research presents evidence in support of the concept that ACF are biologically heterogeneous and that ACF with higher crypt multiplicity are more advanced and stable lesions.

Aberrant crypt foci are used as biological end points in the study of modulators of colon carcinogenesis. A great deal of effort is being devoted to the use of ACF system to identify potential chemopreventive agents in the rodent model. The ultimate goal of many such studies is to apply the information obtained from the rodent studies to the human situation. Identification of aberrant crypts in human colons has strengthened the use of ACF in the rodent model.

Bile acids have been proposed to play an important role in human colon carcinogenesis. This proposal has been tested and to some degree validated in animal studies. The mechanism which has been advocated to play a central role in mediating bile effect is the

ability of bile acids to increase cell proliferation. Studies documented in this thesis cast doubt on this mechanism especially in mediating the effect of bile acids as tumor promoters.

It is recognized that the understanding of the disease process in the whole organism is essential in order to control the development of the disease. The present research has provided additional information on the biological nature of ACF as preneoplastic lesions in the colon and their response to a colon tumor promoter. This information will be valuable in the design of future studies investigating the disease process as well as modulators of colon carcinogenesis.

Further relevance of this research lies in the fact that colon cancer is a major cause of death in North America. It remains uncertain as to what factors are most important in its development or modulation. Research that contributes to a better understanding of this disease is valuable.

C. Future Perspectives

The information generated in this dissertation provides a framework for future colon cancer studies.

In view of the main focus of this work, it is important to elaborate on development of a model which allows for evaluation of the stepwise process of colon carcinogenesis. A better

understanding of the model requires a better understanding of the biological nature of the disease process and intermediate lesions. Although there is mounting information with regard to the response of ACF to modulators of colon cancer, it is still unclear as to what mechanisms are at work in these modulations. Future studies should be devoted to the identification of reliable risk markers and the understanding of cellular and molecular mechanisms involved in the development and growth of ACF. If the ACF model can be further exploited more information regarding the initiation, promotion, and progression stages of cancer development may be revealed.

Chapter IX

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Chapter X

APPENDICES

A. Composition of AIN-76 Mineral and Vitamin Mixtures

AIN-76 Mineral Mixture

Composition:	grams
calcium phosphate dibasic	500.00
sodium chloride	74.00
potassium citrate monohydrate	220.00
potassium sulphate	52.00
magnesium oxide	24.00
maganese carbonate (43-48% Mn)	3.50
ferric citrate (16-17% Fe)	6.00
zinc carbonate (70% ZnO)	1.60
cupric carbonate (53-55% Cu)	0.30
potassium iodate	0.01
sodium selenite	0.01
chromium potassium sulphate	0.55
sucrose, finely powdered	118.00

AIN-76 Vitamin Mixture

Composition:	per kg of mixture
thiamine hydrochloride	600.0 mg
riboflavin	600.0 mg
pyridoxine hydrochloride	700.0 mg
nicotinic acid	3.0 g
D-calcium pantothenate	1.6 mg
folic acid	200.0 mg
D-biotin	20.0 g
cyanocobolamin (vitamin B_{12})	1.0 mg
retinyl palmitate (vitamin A) pre-mix (250,000 IU/g)	1.6 g
DL-α-tocopherol acetate (vitamin E) pre-mix (250 IU/g)	20.0 g
cholecalciferol (vitamin D_3) (400,000 IU/g)	250.0 mg
menaquinone (vitamin K)	5.0 mg
sucrose, finely powdered	972.9 g

B. Anti-Bromodeoxyuridine Staining Procedure

Immunoperoxidase Procedure

1. Depariffinize slides:
   xylene: 5 min - repeat 3x in fresh xylene
   100% ethanol: 2 min - repeat 2x
   95% ethanol: 2 min
   70% ethanol: 2 min
   dH_2O: 5 min

2. Hydrolyse slides in 2N HCl for 1.5 hr.

3. Place in 0.1M $Na_2B_4O_7$ for 5 min to stop reaction.

4. Wash slides in phosphate buffered saline (PBS) 3x. Carefully blot excess solution from the slide and around the tissue.

5. Add signet kit reagent #2 to tissue (normal goat serum blocking agent). Place in humid chamber for 20 min at ambient temperature.

6. Wash slides in fresh PBS 3x. Blot.

7. Add anti-BrUrd Moab (1:40 dilution). Place in humid chamber for 1 hr at ambient temperature.

8. Wash slides in fresh PBS 3x. Blot.

9. Add signet kit reagent #4 to tissue (linking agent - goat anti-mouse Ab). Place in humid chamber for 20 min at ambient temperature.

10. Wash slides in fresh PBS 3x. Blot.

11. Add signet kit reagent #5 to tissue (labelling agent - mouse IgG peroxidase). Place in humid chamber for 20 min at ambient temperature.

12. Wash slides in fresh PBS 3x (no need to blot).

13. 3,3′ Diaminobenzidine Tetrahydrochloride (DAB) staining:
   a. add 1.25 ml of stock DAB to 200 ml PBS.
   b. add 125 μl 30% H_2O_2 and immediately pour over slides.
   c. immerse slides for 5 min.

14. Rinse slides in dH_2O 2x.

15. Dip or immerse slides in haematoxlin stain for 3 sec.

16. Rinse slides in dH_2O 2x.

17 Dehydrate slides:
70% ethanol - 1 min.
95% ethanol - 1 min.
100% ethanol - 1 min.
xylene - 2 min.

18. Apply permount and cover glass.

C. Anti-Proliferating Cell Nuclear Antigen Staining Procedure

Immunoperoxidase Procedure

1. Dehydrate slides:
   xylene: 5 min - repeat 3x in fresh xylene
   100% ethanol: 2 min - repeat 2x
   95% ethanol: 2 min
   70% ethanol: 2 min
   dH_2O: 5 min

2. Hydrolyse slides in 2N HCl for 1.5 hr.

3. Place in 0.1M $Na_2B_4O_7$ for 5 min to stop reaction.

4. Wash slides in phosphate buffered saline (PBS) 3x. Carefully blot excess solution from the slide and around the tissue.

5. Add signet kit reagent #2 to tissue (normal goat serum blocking agent). Place in humid chamber for 20 min at ambient temperature.

6. Wash slides in fresh PBS 3x. Blot.

7. Add anti-PCNA Moab (1:40 dilution). Place in humid chamber for 1 hr at ambient temperature.

8. Wash slides in fresh PBS 3x. Blot.

9. Add signet kit reagent #4 to tissue (linking agent - goat anti-mouse Ab). Place in humid chamber for 20 min at ambient temperature.

10. Wash slides in fresh PBS 3x. Blot.

11. Add signet kit reagent #5 to tissue (labelling agent - mouse IgG peroxidase). Place in humid chamber for 20 min at ambient temperature.

12. Wash slides in fresh PBS 3x (no need to blot).

13. 3,3′ Diaminobenzidine Tetrahydrochloride (DAB) staining:
   a. add 1.25 ml of stock DAB to 200 ml PBS.
   b. add 125 μl 30% H_2O_2 and immediately pour over slides.
   c. immerse slides for 5 min.

14. Rinse slides in dH_2O 2x.

15. Dip or immerse slides in haematoxlin stain for 3 sec.

16. Rinse slides in dH_2O 2x.

17 Dehydrate slides:
70% ethanol - 1 min.
95% ethanol - 1 min.
100% ethanol - 1 min.
xylene - 2 min.

18. Apply permount and cover glass.

D. Data

1. Chapter 4

Table 18. PCNA Labelled cells[1] from colons of animals consuming 0.0%, 0.025%, 0.05%, or 0.1% CHA diet.

Mid section[2]

DIET	Crypt Height	# Labelled Cells	Labelling Index	Size of Prolif. Zone
CO	34.8 ± 1.1^{a}	6.8 ± 0.6^{b}	9.9 ± 1.1^{a}	6.8 ± 0.6^{b}
0.025%CHA	36.5 ± 1.8^{a}	8.8 ± 1.0^{ab}	12.5 ± 1.7^{a}	8.8 ± 1.0^{ab}
0.1%CHA	38.2 ± 1.3^{a}	9.9 ± 0.4^{a}	13.3 ± 1.1^{a}	9.9 ± 0.4^{a}

[1]Values expressed as means ± SEM. Means within a column not sharing a common superscript are significantly different ($p \leq 0.05$).
[2]cm 5-6 from the rectal end of the colon.

2. Chapter 6

Table 19. Effect of CO, CHA, or CHA-delay diet on the number of cells exhibiting proliferating cell nuclear antigen in colonic epithelium[1].

Rectal end[2]

GROUP	CRYPT HEIGHT	# LABELLED CELLS	LABELLING INDEX	SIZE OF PROLIF. ZONE
CO	35.7 ± 2.5[a]	6.6 ± 0.3[a]	9.4 ± 0.7[a]	13.2 ± 0.7[a]
CHA	36.7 ± 1.3[a]	8.1 ± 1.2[a]	11.0 ± 1.3[a]	15.6 ± 2.4[6]
CHA-DELAY	30.2 ± 1.0[b]	6.7 ± 0.6[a]	11.0 ± 0.8[a]	10.7 ± 0.9[a]

Mid section[3]

CO	29.6 ± 1.1[b]	6.7 ± 0.6[a]	11.0 ± 0.51	13.3 ± 0.6[ab]
CHA	45.1 ± 2.5[a]	8.6 ± 2.14	9.4 ± 2.2[a]	18.7 ± 3.2[a]
CHA-DELAY	35.8 ± 2.8[b]	6.3 ± 0.6[a]	8.8 ± 0.3[a]	12.4 ± 1.7[b]

[1]mean SEM. 4 animals/group.
[2]cm 1-2 from the rectal end of the colon.
[3]cm 7-8 from the rectal end of the colon.
[ab]Means within a column with different superscripts differ at $p \leq 0.05$

Table 20. Size of ACF in colons of animals consuming CO, CHA, or CHA-delay diet[1].

[1]For the following tables:
Values expressed as mean ± SEM.
Values within a row with different superscripts differ at $p \leq 0.05$.

WEEK 3

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	31.3 ± 6.6^a	6.3 ± 0.9^b	11.0 ± 3.0^b
3-4	25.3 ± 3.4^a	4.3 ± 1.7^b	5.0 ± 2.3^b
5-6	10.0 ± 3.2^a	0.3 ± 0.3^b	0.3 ± 0.3^b

WEEK 6

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	49.7 ± 15.5^a	22.0 ± 5.0^a	13.7 ± 6.5^a
3-4	49.3 ± 15.9^a	13.3 ± 2.8^b	10.7 ± 1.8^b
5-6	35.3 ± 11.2^a	7.0 ± 4.6^b	2.3 ± 0.3^b
7-8	9.3 ± 2.9^a	1.3 ± 0.9^b	0.0 ± 0.0^b
9-10	7.0 ± 2.9^a	1.0 ± 0.6^b	0.0 ± 0.0^b

WEEK 9

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	49.3 ± 16.3[a]	34.7 ± 16.7[a]	30.0 ± 4.0[a]
3-4	51.7 ± 8.6[a]	26.3 ± 5.7[b]	23.0 ± 5.7[b]
5-6	40.3 ± 8.1[a]	12.7 ± 7.3[b]	15.3 ± 5.7[b]
7-8	13.0 ± 1.2[a]	3.0 ± 2.0[b]	3.0 ± 1.5[b]
9-10	10.0 ± 1.2[a]	2.7 ± 2.2[b]	1.3 ± 0.3[b]

WEEK 12

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	26.7 ± 10.1[a]	19.3 ± 3.8[a]	25.3 ± 7.5[a]
3-4	41.3 ± 13.4[a]	37.0 ± 14.3[a]	47.0 ± 18.3[a]
5-6	27.7 ± 7.1[a]	20.0 ± 4.6[a]	27.3 ± 12.3[a]
7-8	8.3 ± 5.0[a]	6.0 ± 2.1[a]	6.0 ± 3.0[a]
9-10	8.0 ± 2.9[a]	10.3 ± 5.2[a]	1.7 ± 0.9[a]

WEEK 15

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	42.3 ± 14.3[a]	15.3 ± 2.3[a]	18.7 ± 4.9[a]
3-4	48.3 ± 14.5[a]	25.3 ± 8.1[a]	23.7 ± 5.8[a]
5-6	30.3 ± 8.4[a]	10.7 ± 3.2[b]	22.0 ± 2.5[ab]
7-8	10.7 ± 0.3[a]	6.7 ± 4.3[a]	5.0 ± 1.7[a]
9-10	4.7 ± 2.9[a]	4.7 ± 2.7[a]	8.3 ± 0.7[a]

WEEK 18

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	39.3 ± 10.5[a]	10.0 ± 2.9[b]	13.3 ± 3.0[b]
3-4	71.0 ± 2.5[a]	16.7 ± 4.7[b]	26.0 ± 9.1[b]
5-6	51.0 ± 9.6[a]	12.0 ± 3.8[b]	16.0 ± 6.2[b]
7-8	21.0 ± 3.1[a]	5.3 ± 0.7[b]	10.0 ± 6.2[ab]
9-10	14.7 ± 2.8[a]	2.7 ± 0.3[b]	6.3 ± 1.8[b]
11-12	7.3 ± 3.8[a]	0.7 ± 0.7[a]	2.7 ± 1.2[a]

WEEK 21

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	44.7 ± 8.7[a]	20.7 ± 7.8[a]	21.3 ± 3.3[a]
3-4	61.7 ± 11.6[a]	26.3 ± 5.8[b]	25.3 ± 7.8[b]
5-6	45.7 ± 10.7[a]	17.7 ± 1.9[a]	19.0 ± 8.1[a]
7-8	14.7 ± 3.3[a]	8.0 ± 3.6[a]	4.7 ± 2.3[a]
9-10	16.7 ± 4.4[a]	6.0 ± 0.6[a]	7.3 ± 3.8[a]
11-12	5.0 ± 1.2[a]	3.7 ± 1.2[a]	1.7 ± 1.2[a]

WEEK 24

X $10^{-2}mm^2$	CO DIET	CHA DIET	CHA-DELAY
1-2	45.3 ± 1.9[a]	11.7 ± 0.9[b]	15.7 ± 1.9[b]
3-4	44.3 ± 5.2[a]	29.3 ± 7.3[a]	27.3 ± 3.8[a]
5-6	36.3 ± 7.8[a]	22.0 ± 2.3[a]	19.7 ± 4.8[a]
7-8	11.7 ± 1.8[a]	8.3 ± 2.6[a]	7.3 ± 1.3[a]
9-10	11.7 ± 2.6[a]	8.3 ± 1.3[a]	5.3 ± 2.7[a]
11-12	10.0 ± 3.1[a]	3.0 ± 1.0[ab]	2.0 ± 1.5[b]