COLONIC CRYPT CELL DYNAMICS AS PREDICTORS OF NEOPLASTIC POTENTIAL

A Thesis

by

Laurence T. Glickman, B.A., M.D.C.M.

Submitted to the

Faculty of Graduate Studies and Research

in partial fulfilment of

the requirements for the degree of

MASTER OF SCIENCE

IN

EXPERIMENTAL SURGERY

McGill University

Montreal

July 1985

TABLE OF CONTENTS

TO JUDY

ABSTRACT

The proliferative characteristics and nuclear aberrations of the colonic crypt cells in the dimethylhydrazine (DMH) sensitive A/J mouse and DMH resistant C57BL/6J mouse were examined before and after a single exposure to the carcinogen. The untreated A/J mouse was found to have a longer crypt column, a higher labelling index and a wider proliferative compartment than the C57BL/6J mouse. Baseline nuclear aberrations were similar in both strains. After acute exposure to DMH there is an initial drop in proliferation followed by an overshoot and a subsequent return to baseline. Nuclear aberrations peaked at 12 hours then returned to baseline. These post-DMH patterns of proliferation and cell loss and recovery over time were similar and parallel in both strains. The data suggests that the susceptibility to DMH carcinogenesis can be predicted by the indigenous number and distribution of DNA synthesizing cells in the murine colonic mucosa and that the nuclear aberration index assay is a good indicator of carcinogen exposure.

RÉSUMÉ

Nous avons étudié l'effet d'une dose unique du carcinogène 1,2-diméthylhydrazine (DMH) sur les caractéristiques prolifératives et les anomalies nucléaires des cellules appartenant aux cryptes du colon chez la souris. Deux souches différentes de souris furent utilisées: la souche sensible A/J et la souche résistante à la DMH C57BL/6J. Tous les paramètres furent measurés avant et après que la dose unique de DMH ait été administrée. Avant l'utilisation de DMH, les souris A/J avaient de plus longues cryptes, un compartiment prolifératif plus large et un indice de marquage plus élevé que les souris de souche C57BL/6J. Les anomalies nucléaires de bare étaient comparables pour les deux souches. Après l'utilisation de la DMH, on observa initialement une baisse de l'indice prolifératif, suivie d'une compensation exagérée, puis d'un retour aux valeurs de base. Les anomalies nucléaires culminèrent vers 12 heures post-injection, pour retomber par la suite aux valeurs de base. Les deux souches de souris suivirent un schéma similaire et parallèle de prolifération et de récupération et perte cellulaire après l'utilisation de la DMH. Ces résultats suggèrent que l'on peut prédire la susceptibilité au carcinogène DMH par l'étude du nombre et de la distribution des cellules muqueuses du colon qui synthétisent de l'ADN chez la souris; et que l'indice d'anomalie nucléaire reflète adéquatement l'exposition à un carcinogène.

FOREWORD

This work was performed at the University Surgical Clinic, The Montreal General Hospital, McGill University between July 1, 1984 and June 30, 1985 under the supervision and guidance of Dr. David M. Fleiszer.

This thesis is divided into three separate parts. The first part is a brief review of the literature and includes sections on the epidemiology and etiology of colon cancer. The genetic and kinetic contributions to this disease are then covered in greater detail because they provide the foundation on which the experimental work is based.

The second part includes the methodology, results and discussion of two experiments carried out during this year. The first experiment entitled "The Proliferative Characteristics of Colonic Crypt Cells as Predictors of Subsequent Tumor Formation" has been submitted to Cancer Research for publication, and the abstract from this paper has been accepted for presentation at the 71st Annual Clinical Congress of the American College of Surgeons in October 1985 and will be published in the Surgical Forum, Volume 36.

An abstract from the second experiment entitled "1,2-Dimethylhydrazine Induced Nuclear Aberrations in C57BL/6J and A/J Mouse Colonic Crypts" has been submitted to The Association for Academic Surgery for presentation at their 19th Annual Meeting in November 1985.

Because of the nature of this work, some of the discussion and conclusions drawn from these two experiments are repeated, and for this overlap I apologize.

Part three includes the final summary and discussion and the

references of the thesis.

During the course of this year in the laboratory I have learned a great deal about this enigmatic disease called colon cancer. The goal of the scientist and non-scientist interested in colon cancer must be the prevention and early detection of this disease. I hope that in some manner this small work makes a contribution towards that end.

Laurence T. Glickman, M.D.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, teacher and friend Dr. David M. Fleiszer for having facilitated this work with his advice, criticisms, encouragement and enthusiasm throughout this year in the laboratory.

I would also like to thank Dr. Emil Skamene for his support and insights, Dr. Samy Suissa for his excellent statistical analysis of the data, and Dr. Mary K. Senterman for her help and enthusiasm in analyzing the histological specimens. The constructive critiques and advice of Dr. Ray Chiu are also deeply appreciated.

The expert technical assistance of Ms. Mary Luponio, Carolyne Luty and Wynanne Bindon and the typing skills of Ms. Maria Archina and Ms. Brenda Bewick are also gratefully acknowledged.

The superb autoradiographs were prepared by Dr. Beatrice Kapriwa and Mr. Fernando Evaristo of the Department of Anatomy, McGill University.

As well I must thank the Audiovisual Department at The Montreal General Hospital, in particular Ms. Ildiko Horvath, for the superb illustrations.

Finally I want to thank my wife, Judy Lighter, whose caring, support and love throughout the past 12 months has been a great source of inspiration to me. It is to Judy that I dedicate this thesis.

ABBREVIATIONS

A	-	A/J Mouse (mice)
B	-	C57BL/6J mouse (mice)
DMH	-	1,2-Dimethylhydrazine
LI	-	Labelling Index (indices)
MF	-	Mitotic Figure(s)
MI	-	Mitotic Index (indices)
NA	-	Nuclear Aberration(s)
NAI	-	Nuclear Aberration Index (indices)
PC	-	Proliferative Compartment(s)
TBW	-	Total Body Weight

COLONIC CRYPT CELL DYNAMICS AS PREDICTORS OF NEOPLASTIC POTENTIAL

PART I

1. EPIDEMIOLOGY OF COLON CANCER

Cancer of the large intestine is a disease of the industrialized countries of Europe, Australia and North America (1-3). In Canada and the United States, colon cancer represents one of the largest cancer problems when considering both sexes together. Approximately 3% of all deaths in North America are due directly to this disease and 25/100,000 cases or about 15% of all malignancies in men and women are located in the large bowel, and 5% of all men and women in North America will develop this disease before the age of 75 (4, 5). Females are affected by colonic cancer more than males in a ratio of 1.2:1. Incidence increases with age peaking in the seventh decade, but 8% of cases are diagnosed before age 40. Thus colon cancer represents a serious health problem and over the last two decades there has been an increasing interest in this disease. Despite volumes of epidemiologic and experimental data on colon cancer, very little is known about the cause, prevention, early detection or cure of colon cancer.

2. ETIOLOGY OF COLON CANCER

(A) Human Studies

In the last 15 years clinicians and scientists have shed much light on the etiological roles of diet and fecal factors in colonic carcinogenesis. Migration studies have demonstrated that genetically homogeneous populations moving from their low risk country of origin to North America significantly increase their risk of developing colon cancer (6, 7). The emphasis has been on correlation studies and group comparisons between the intake of animal proteins, meat, fat and fiber, and the risk of developing colon cancer.

Several studies have found a high correlation between colon cancer mortality and the intake of animal proteins, meats and fat (8-10). These studies were supported by observations of the Seventh Day Adventists who, being lacto-ovo vegetarians with a low fat, high fiber intake, have a very low incidence of colon cancer (11, 12).

Dietary fiber and "roughage" have been hypothesized to play a protective role in the pathogenesis of colon cancer (13). These protective mechanisms are thought to be mediated by fiber's effects on intestinal transit time, stool bulk, and intestinal microflora (13), as well as its effects on binding and metabolism of possible carcinogens (14).

Fecal characteristics have been implicated in the colon carcinogenic process as well. Hill has suggested that fecal bile acid concentration is determined by fat intake and that carcinogenic risks are linked to the concentration of secondary bile acids in the feces. These secondary bile acids are produced in the large bowel under the influence of a bacterial flora high in clostridia of the dehydrogenating type (15-18).

Similarly, analysis of fecal samples obtained from various populations exhibiting different risks of developing colon cancer, or from patients with established colon cancer have attempted to identify a specific carcinogenic species of bacteria. Human fecal flora is extremely complex and contains more than 200 organisms from 40 different species of bacteria (19). No consistent pattern has been established in the profiles of these samples, and there is no correlation between the presence of particular organisms in the stools of colon cancer patients, or in those patients thought to be predisposed to the disease. Thus no specific pathogenic role for any particular bacterial species has been defined for colon cancer (19). Therefore in the absence of direct information on specific bacteria, deductions must be made from indirect sources. As most known carcinogens require metabolic activation in order to express their activity, it has been hypothesized that fecal bacteria may contribute to colon carcinogenesis by activating benign substances in situ. This activation is thought to yield direct acting carcinogens, tumor promoters and cocarcinogens or precursor species that may be further metabolized to active species in the target tissue (20).

(B) Animal Studies

The epidemiologic and correlation studies are important and provide a great deal of information regarding the etiological contributions of diet and fecal factors in colonic carcinogenesis. They are difficult to interpret, however, because they are fraught with poor or no controls, and they often yield conflicting results. In order to bypass these obstacles, an experimental model of colon carcinogenesis was developed. The experiments conducted by Drukrey

demonstrated that both oral and subcutaneous administration of 1,2-Dimethylhydrazine (DMH) induces tumors in rat colons at a high rate of incidence (21). Subsequently, it was shown that DMH was also capable of inducing colonic neoplasms in mice (22). At present the intestinal tumors induced by DMH and its metabolites, azoxymethane and methylazoxymethanol are the most popular experimental models used in studies on different aspects of the colonic carcinogenic process.

An important feature of DMH colonic carcinogenesis is that the tumors that develop mimic human neoplasms regarding their anatomic location in the gastrointestinal tract, with a preponderance of tumors in the descending colon. As well, there is a close similarity in pathologic features from early focal atypias and hyperplasias, to adenomatous polyps and adenocarcinomas (23).

Thus the DMH model of colon carcinogenesis has served to explore and separate out the interacting endogenous and exogenous etiologic factors of this complex disease. This model provides a unique tool for systematic studies of the risk factors observed in the human setting and for determining whether or not their suspected associations can actually be reproduced under highly controlled laboratory conditions.

The DMH model has been used extensively to examine the effects of fat, fiber and protein on colon carcinogenesis (24). It has repeatedly been shown that fiber and its components have protective effects (25, 26) and dietary fat and meat protein have promotive effects on DMH-induced colon cancer (27-29). The role of bile acids in colon carcinogenesis have also been examined experimentally (30, 31). The data from these studies suggest that bile acids are not carcinogens per se, but appear to act as promoters of large bowel

carcinogenesis.

As previously noted, the role of human fecal flora in the etiology of large bowel cancer is poorly defined because of the complexity of fecal flora and their breakdown products. Attempts have been made at examining rodent fecal bacteria and their roles in DMH carcinogenesis. The incidence of colon tumors in DMH-treated germ-free rats is lower than in conventional rats (24, 31). It has also been demonstrated that oral erythromycin or tetracycline can significantly reduce DMH-induced tumorogenesis (32). These results are felt to be due to the suppressing effects of the antibiotics on the metabolic activity of the intestinal flora. More recent data using metronidazole (33, 34), and neomycin (35) on DMH carcinogenesis suggest that certain antibiotics may result in increased excretion of fecal cholesterol and bile acids, and possibly decreased bacterial β-glucuronidase activity, resulting in more tumors in antibiotic treated animals than in control animals. The only conclusion that can be drawn from these studies is that the definitive contribution of fecal microflora in the colonic carcinogenic process remains elusive and unresolved.

In summary, on the basis of variations in incidence of colon cancer in different regions of the world, in view of the altered cancer risk of migrant populations and on the basis of data provided by animal studies on DMH carcinogenesis, it has been accepted that diet is a major etiologic factor in the genesis of colon cancer.

3. GENETICS OF COLON CANCER

(A) Human Studies

Although environmental factors are considered the major determinants in the development of colon cancer in man, genetic factors have also been implicated in susceptibility to spontaneous and induced colonic tumors (36, 37). Hill has pointed out that despite there being a good correlation between the incidence of colon cancer and diet in population studies, the results of investigations of individuals in case-control studies have been equivocal (38). Thus although environmental factors are important in determining incidence of colon cancer in a population, it appears to be much less so in determining the risk of an individual. The roles of genetic variables appear to be heterogeneous, embracing different etiologic combinations. Simple Mendelian modes of genetic transmission have been associated with several disorders that involve the colon. These include Familial Polyposis Coli, Gardner's Syndrome, Peutz-Jeghers Syndrome and Juvenile Polyposis Coli. All these disorders are characterized by their autosomal dominant mode of inheritance. In Familial Polyposis Coli patients, large numbers of colorectal adenoma are present in early adulthood and if left untreated, their risk of developing colorectal cancer is virtually 100% (39). Gardner's Syndrome is a variant of Familial Polyposis Coli and is characterized by mandibular osteomas and multiple epidermal cysts. Other possible associated lesions include thyroid cancer, carcinoma of the Ampulla of Vater, duodenum and gall bladder (40). In Peutz-Jeghers Syndrome pigmented lesions appear on the lips and buccal mucosa and also on the dorsum of the hands and feet. These tumors are considered hamartomas and have a low potential for malignant transformation (41).

Distribution of the polyps in Juvenile Polyposis is not as extensive as in Familial Polyposis, and are seen primarily in the large intestine. These lesions are also hamartomatous polyps and usually appear by six years of age. These are also considered to carry a low risk of malignancy (42).

These heritable syndromes that predispose to colonic cancer all have in common an association with multiple polyps of the colon. Lynch has described another colon cancer predisposed group that is not associated with multiple colonic polyps. He has called these the "non-polyposis syndromes", and suggests that in fact, the majority of hereditary colon cancer occurs in this group of patients (43). These "non-polyposis syndromes" include hereditary site specific colon cancer (HSSCC) or Lynch Syndrome I, and the Cancer Family Syndrome (CFS), or Lynch Syndrome II (43, 44). In CFS a high proportion of family members develop large bowel cancer at a young age. As well, CFS is associated with a significant excess of multiple primary tumors, predominantly adenocarcinomas of the endometrium and ovary. Recent studies, therefore, indicate that familial associations of colon cancer are higher than in control groups, suggesting that inherited factors may play a significant role in the genesis of this disease.

(B) Animal Studies

The literature is filled with data on the differences in susceptibility to DMH-induced tumors in different strains of experimental animals. Moon and Fricks (45) found that BD-IX rats were more sensitive to DMH than BD-II rats, and Asano and Pollard (46) reported that Sprague-Dawley rats were more susceptible than Lobund-

Wistar rats. Since Evans' first report on the differential susceptibility to DMH carcinogenesis in mice (47), several reports have confirmed that different inbred mouse strains have varying degrees of sensitivity to the carcinogenic action of DMH (48-53). Hybrids derived from the cross of DMH resistant C57BL/Ha and DMH sensitive ICR/Ha inbred parents revealed that the genes inherited from the ICR/Ha parent were responsible for their sensitivity to the tumor-inducing effects of the carcinogen (48). There was no evidence of sex or Y-group linkage and the inherited sensitivity in the F2 reciprocal backcross hybrids appeared to be inherited in a simple Mendelian dominant fashion.

Thus there is strong evidence that the susceptibility to colonic carcinogenesis both in man and in the experimental animal is to a significant degree, genetically determined.

4. CELLULAR KINETICS IN NORMAL COLONIC EPITHELIUM

Knowledge of the proliferative characteristics in the colonic crypts in the resting state is of great importance in understanding the carcinogenic potential of the colon. In the last thirty years, great strides have been made in recording kinetic data derived from stathmokinetic studies based on autoradiographs of the colon. Much is now known about those factors which regulate cellular proliferation in the colonic epithelium.

Normal cell proliferation in colonic crypts of mammalian species, including man, has been found to occupy the lower two thirds of the glands with the major zone of DNA synthesis in the lower third (54-58). As cells mature they migrate up the sides of the crypts and are eventually shed at the surface epithelium. These characteristics are common both to the rodent and to man. Within the crypts in the descending colon, cell proliferation has been measured by numerous investigators and while the stathmokinetic data derived from these studies are beyond the scope of this thesis, it is worth describing several important variables which are thought to regulate cellular proliferation to a significant degree. Firstly, there is good evidence that colonic crypt cell proliferation has strong circadian rhythms. Therefore estimates of colonic kinetic parameters vary depending on the time of day they are measured (59, 60). Secondly, several studies have shown that colonic kinetics may be affected by steroids. Estrogen has been shown to inhibit DNA synthesis in murine colons (61), and oophorectomized mice contain fewer cells in their crypts (62). Thus the sex of the animal is felt to affect crypt cell proliferation. Finally, diet has also been shown to alter colonic proliferative indices (63-66). This phenomena is thought to relate to

the varying geographic incidences of colon cancer worldwide.

It has been shown that there are also variations in the proliferative activity within the crypts themselves. Cryptal cell proliferation in mice has been investigated by several groups of scientists using a variety of cytokinetic techniques. Lipkin and Quastler (56) found that the labelling index (LI - the LI is the percentage of all cells in any given cell population, that are undergoing synthesis of DNA) was highest in the mid-portion of the crypt, with lower values in the basal cells, and no labelling in the upper part of the crypt. Chang and Leblond (67) found that in the descending murine colon, cells at the base of the crypt had the highest LI when compared to the mid-portion and upper regions of the crypts. This type of distribution was similar to that described by Richards (68) and de Rodriguez et al (58). The tritiated thymidine studies have shown that the lower production rate in the base of the crypt is associated with a long cell cycle time and a high growth fraction, and that as cells migrate up the crypt wall, the cell cycle time shortens and the growth fraction falls. These features of cellular proliferation and growth are schematically illustrated in Figure 1 (adapted from Tutton and Barkla, ref. 69).

In man, approximately fifteen percent of all cells in the lower two thirds of the colon crypt undergo DNA synthesis simultaneously, and during migration of these cells, the number that continue to proliferate decreases as they progress towards the lumenal surface. These cells undergo terminal differentiation within hours, and several studies have shown that cellular migration and replacement of the colonic mucosa takes between 3-8 days in man (57, 70).

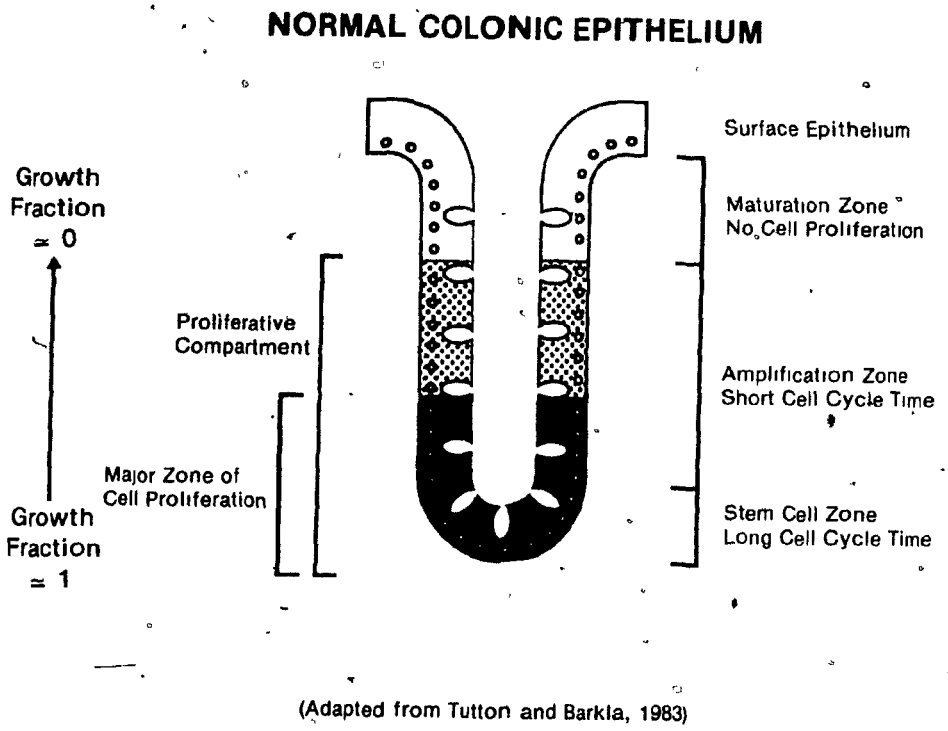

Figure 1. Schematic representation of epithelial cell kinetics in the normal colon.

Different proliferative indices have also been reported along different sites of the large bowel of the mouse (71). In addition site-specific differences in the size or width of the proliferative compartments (PC), as well as the heights of the crypt columns have also been reported in the normal mouse colon (72), where the highest crypt columns and greatest LI appear in the mid-distal colon.

In summary, the measurements of cellular kinetic data in the normal colon has provided the framework for analyzing the growth characteristics of gastrointestinal cells during neoplastic transformation. In the resting state, the major zone of DNA synthesis is in the lower third of the crypt, and as cells mature they migrate up the crypt wall and are ultimately shed into the gut lumen. Several variables including diurnal variations, sex and diet are known to affect cellular proliferation. Finally it has been shown that proliferative parameters vary from site to site along the length of the colon, and also within individual crypts.

5. CELLULAR KINETICS IN NEOPLASTIC COLONIC EPITHELIUM

(A) The Acute Response

Several studies have examined the acute effects of DMH on the kinetics of rodent colonic crypt cells. The initial cytotoxic effects of DMH are to produce necrosis of the cell, within the PC of the crypt and to suppress the LI in these cells as well (73-75). The combination of cell necrosis and suppression of the LI results in a significant reduction of crypt column height and total crypt cell numbers. These changes commence within 6 hours after DMH administration and are reported to peak between 12 and 24 hours. A phase of compensatory regenerative activity is then seen in the next 48 hours and this results in an "overshoot" in the LI and an increase in crypt cell number. Within 5-7 days after the initial carcinogen exposure, the normal mucosal architecture and proliferative activity have been restored. The sequence of these acute changes are summarized in Table 1.

Thus after a single exposure to DMH, the initial cytotoxic effects result in cell death and the suppression of DNA synthesis. The crypt then recovers by increasing its synthesis of DNA and re-establishing its baseline kinetic activity.

(B) The Chronic Response

Measurements of cellular proliferation patterns during ongoing chemical carcinogenesis have also provided important information regarding the growth characteristics of colonic cells during neoplastic transformation. Many of the changes observed in human colon specimens are similar to those that occur in rodent colons. Table 2 summarizes the chronic response of the rodent colonic epithelium to DMH carcinogenesis.

Table 1

CELL KINETICS IN DMH-TREATED COLON EPITHELIUM

The Acute Response - Cell Death and Recovery

Time Post-DMH Exposure (Hours)	Response
6-24	1. Drop in crypt column height
	2. Drop in labelling index
	3. Drop in cell population
24-72	4. Compensatory, proliferative response
	5. "Overshoot" in labelling index
72-120	6. Increase in crypt cell population
	7. Return to baseline indices

Table 2

CELL KINETICS IN DMH-TREATED COLON EPITHELIUM

The Chronic Response - Mucosal Hyperplasia to Anaplasia

Time Post-DMH Exposure (Weeks)	Response
2-6	1. Increase in crypt column height
	2. Increase in crypt column number
	3. Increase in total cell population
	4. Increase in cell production rate
	5. Extension upwards of the proliferative zone
	6. Increase in the labelling index
6-15	7. Migrational defects
	8. Mucosal atypias
	9. Mucosal dysplasia
->15	10. Adenomatous polyps
	11. Ca in situ
	12. Adenocarcinomas

The primary site of activity and response to DMH is in the distal rodent colon, which is analogous to the human situation. As early as two weeks after DMH treatment, both an increase in the number of crypt columns and an increase in crypt column height are seen (76, 77). This results in an increase in total crypt cell number. These hyperplastic changes are temporary if DMH treatment is discontinued. However, Richards has shown that in mice given eight or more injections of DMH, the hyperplastic changes persist and may continue and progress to gross carcinomas (77).

Several investigators have noted that there is an increase in the cell production rate in the middle regions of the crypts following chronic DMH exposure, and this is accompanied by an increase in the LI in this zone (68, 75, 78, 79). Studies on the distribution of labelled cells in microscopically normal appearing mucosa, in animals treated with several doses of DMH have revealed that there is a widening of the PC (22, 75, 80, 81). This alteration in the distribution of labelled cells in a region which normally contains non-dividing, differentiated cells, is thought to result from disturbances in the control of proliferation regulation. Although poorly understood, this mechanism normally suppresses DNA synthesis in this zone (23, 82).

The extension of the proliferative zone towards the upper third of the crypt and along the lumenal surface, but with the lower third, still the major zone of DNA synthesis has been termed a "Stage 1 Abnormality" by Deschner (82). This abnormality has been reported in patients with polyps, familial polyposis and colon cancer (82-84). A second abnormality has also been described which is seen in the normal

appearing mucosa of patients with established colon cancer. This "Stage 2 Abnormality" involves an upward shift of the major zone of proliferation towards the middle and upper portions of the crypts. Thus in a Stage 2 Abnormality DNA is primarily synthesized in the upper two thirds of the glands (85).

In summary, there are several important morphological and proliferative changes that occur in the colonic crypts of both rodent and man during the neoplastic process. These include an increase in the total cell population of the crypts, an increase in the cell production rates in the middle third of the glands, an upward extension of the PC and an overall increase in the LI. These abnormalities are summarized and schematically illustrated in Figure 2 (adapted from Deschner, ref. 82).

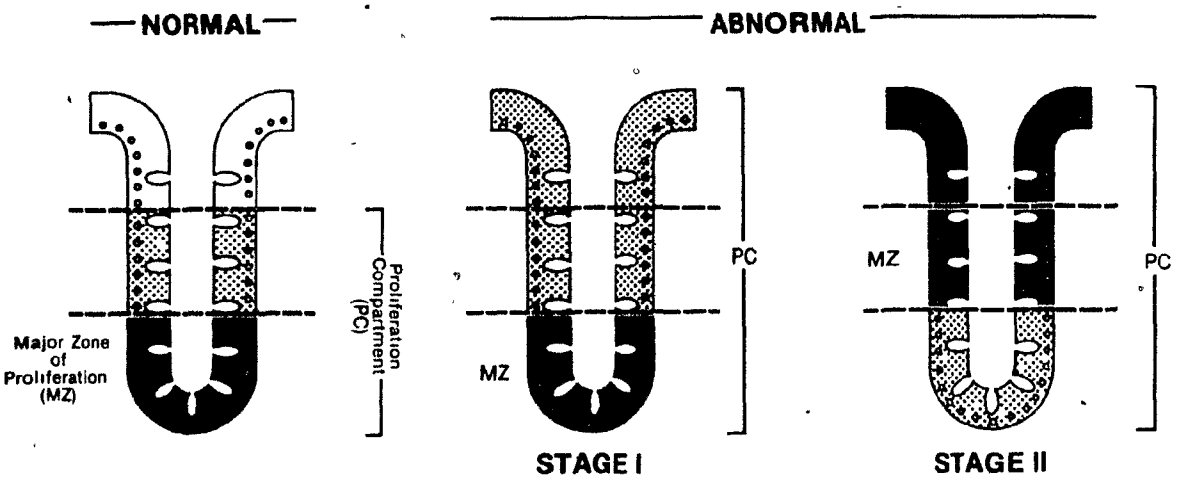

Figure 2. Schematic representation of three types of histologically normal appearing crypts found in colonic mucosa of cancer patients and DMH treated animals. On the left, a crypt with normal epithelial cell proliferation. In the middle, the Stage I Abnormality, and on the right, the Stage II Abnormality.

6. NUCLEAR ABERRATIONS IN COLONIC EPITHELIUM

It has recently been shown that there is a strong correlation between the mutagenic and carcinogenic properties of various chemical carcinogens on several tissues in the rodent and in man (86, 87). The colonic epithelium is one such tissue which has been shown to be extremely sensitive to the mutagenic effects of both radiation and colonic carcinogens (87-89). A short-term test for genotoxicity has been proposed by several investigators (87, 90) and they have demonstrated that it is possible to assess the relationship between carcinogenic dose and response by means of quantifying micronuclei and related nuclear abnormalities in colonic crypt cells following carcinogenic exposure. Maskens first demonstrated that the proportion of abnormal nuclei (in the form of karyorrhexis) following DMH exposure, correlated well with the sensitivity of individual segments in the rat colon to eventual DMH-induced tumor development (91). It was subsequently shown that non-colon carcinogens and non-carcinogens did not induce these nuclear aberrations (NA) (87, 90). Thus it is felt that the quantification of cryptal NA is a sensitive and specific assay for colonic carcinogen exposure.

Three principle varieties of nuclear abnormalities have been described. Pyknotic and karyorrhectic nuclei are characteristic of the process of apoptosis (cell death) and are heralded by nuclear condensation (pyknosis), followed by nuclear fragmentation (karyorrhexis) and finally by phagocytosis by adjacent epithelial cells, or extrusion into the crypt lumen (92). Micronuclei are thought to arise from acentric chromosomal fragments that, lacking a spindle attachment site, are often outside the reforming nucleus of daughter cells (93), or from whole chromosomes as a result of damage

to cellular DNA (94). Recently it has been suggested that micronuclei do not constitute a significant fraction of all NA seen after carcinogen exposure (95). Therefore NA may be primarily a manifestation of the apoptotic process.

Thus following DMH exposure, at the molecular level, nuclear macromolecules are alkylated which leads to pyknosis, karyorrhexis and damage to DNA. This might then lead to chromosomal anomalies, genetic mutations and altered gene expression. It has therefore been proposed that the characterization and quantification of NA might be used as an index for carcinogen exposure, and possibly as a biological marker for neoplastic growth potential.

7. PURPOSE OF THIS STUDY

One major goal of the surgical and gastrointestinal oncologist is to develop new techniques to help reduce the incidence of colon cancer, and to increase the survival rate of this disease. The three most commonly used modalities for colon cancer detection are: the hemoccult blood test, the double contrast barium enema, and fiberoptic colonoscopy. Although sensitive, all of these techniques diagnose advanced disease. Ideally, a sensitive, specific and technically simple method of colon cancer screening should be developed. The aim of the following experiments is to provide some basic foundations for such a method. Hopefully preneoplastic changes in the proliferative patterns of the colonic epithelium could then be detected, and individuals or populations at risk for the subsequent development of colon cancer would be identified.

The proliferative characteristics of the normal and neoplastic colonic crypt cells in the rodent and in man, and the genetic contributions to this disease have been described. In examining the acute effects of the exogenous colon carcinogen DMH on the endogenous colonic crypt cell proliferative patterns in two genetically homogeneous inbred strains of mice, the following are the objectives of this project.

In experiment 1:

1) to quantify the baseline proliferative indices of the distal colonic crypts in two genetically distinct groups of mice that have known dissimilar sensitivities to the long-term effects of DMH.
2) to document the sequence of changes that occur in the crypts of each strain following a single exposure to DMH.

3) to demonstrate that the sensitivity to DMH carcinogenesis can be predicted by the indigenous number and distribution of DNA synthesizing cells in the distal colonic mucosa.

In experiment 2:

1) to quantitate the baseline absolute and relative numbers of aberrant nuclei and mitotic figures in the distal colonic crypts of two genetically distinct strains of mice with known dissimilar sensitivities to the long-term effects of DMH.
2) to document the number and distribution of aberrant nuclei in the crypts of each strain following a single exposure to DMH.
3) to evaluate the nuclear aberration index assay as an indicator of acute DMH exposure.
4) to evaluate the nuclear aberration index assay as a predictor of neoplastic growth potential.

PART II

EXPERIMENT 1

THE PROLIFERATIVE CHARACTERISTICS OF COLONIC CRYPT CELLS AS PREDICTORS OF SUBSEQUENT TUMOR FORMATION

8. THE PROLIFERATIVE CHARACTERISTICS OF COLONIC CRYPT CELLS AS PREDICTORS OF SUBSEQUENT TUMOR FORMATION

(A) INTRODUCTION

1,2-Dimethylhydrazine (DMH) has been shown to be a reliable inducer of colonic tumors in mice and rats (21, 22). Much of the recent experimental research has concentrated on the effects of manipulating diet on DMH carcinogenesis (25, 26, 28). The dietary effects are thought to be mediated through changes in the intestinal microflora and in the composition of bile acids and neutral sterols secreted in the intestine (15-20, 30, 31). Although these environmental factors are thought to be the major determinants in the development of colon cancer in humans, genetic factors have also been implicated in susceptibility to spontaneous and induced colonic tumors (36-44). Since Evans' first report on the differential susceptibility of several mouse strains to DMH-induced tumors (47), several studies have confirmed that DMH sensitivity is strain dependent (48-53). The mechanisms by which sensitivity or resistance to DMH is inherited are poorly understood. One approach by which to examine this difference is to assess the early changes that occur in the colonic crypt cells of different inbred strains of mice following a single exposure to the carcinogen. It has been shown that a single subcutaneous or intrarectal dose of DMH can induce specific cytologic changes in the colonic mucosa, as well as a rapid inhibition of DNA synthesis in S-phase cells and an early induction of nuclear aberrations in the PC of the crypts (73-75). These acute changes are thought to reflect the vulnerability of the target tissue to the carcinogen and are site-specific for DMH. In the rodent, it is the distal colon that has the greatest acute cytotoxic and proliferative changes, as well as the

greatest tumor load following chronic DMH carcinogenesis (74).

It has also been suggested that a relationship exists between the proliferative characteristics of the colonic mucosa and the development of neoplasia both in man and in the rodent (84). In view of the likelihood that the difference in strain sensitivity to DMH is genetically controlled, it has been proposed that the LI and distribution of DNA synthesizing cells of the murine colonic crypts in the resting state should be able to predict the degree of sensitivity to ongoing carcinogen exposure (96, 97).

In order to test these theories, DMH was used as the carcinogen and two inbred strains of mice, the DMH-sensitive A/J (A) mouse and the relatively DMH resistant C57BL/6J (B) mouse were used as the test animals. These mice are especially well suited for analysis in view of their great differences in resistance to infection and malignancy (98, 99), and because a large number of phenotypic markers have already been mapped to the genome.

Thus the purpose of this study is to 1) quantify the baseline proliferative indices of the distal colonic crypts in two genetically distinct groups of mice that have different sensitivities to DMH and 2) document the sequence of changes that occur in the crypts of each strain following a single exposure to DMH. The main objective then is to demonstrate that the sensitivity to DMH carcinogenesis can be predicted by the indigenous number and distribution of DNA synthesizing cells in the distal colonic mucosa.

(B) MATERIALS AND METHODS

Female mice of the A and B strains were obtained at 7 weeks of age and were observed for 1 week before the start of the experiment.

Both strains were purchased from the Jackson Laboratory (Bar Harbor, Maine). All mice were fed Purina Lab Chow (Ralston Purina Co., Richmond, Indiana) and water ad libitum, and were housed in wire cages, 4-5 mice/cage with wood chip bedding in temperature, humidity controlled rooms with a 12 hour light/dark cycle.

The DMH exposed animals were given a single subcutaneous injection of DMH (Aldrich Chemicals Corp. Inc., Milwaukee, Wisconsin), at a dosage of 15 mg/kg body weight. Fresh solutions were prepared by dissolving DMH in normal saline at a concentration of 0.3 gm % and alkalinized with NaOH to a pH of 6.5. Groups of 5 animals were sacrificed at 1, 12, 24, 48, 72, and 96 hours following the DMH injection. Six animals in each strain served as controls and received saline injection only (Figure 3).

All animals received an intraperitoneal injection of tritiated thymidine, 1 μCi/gram body weight (specific activity 6.5 Ci/mmol, New England Nuclear Ltd., Lachine, Quebec) 1 hour prior to sacrifice. All animals were killed between 10 and 11 am to avoid diurnal fluctuations in the proliferative indices (59-60).

a) Tissue Handling

All animals were killed by cervical dislocation. The large bowel was removed, opened along its entire length and washed with cold saline solution. The lengths and weights of the colon from anus to cecum were recorded, and then the distal 3 cm of colon was pinned, mucosal surface upward, to a cork board and fixed in 10% buffered formaldehyde solution for 24 hours.

b) Autoradiographic Slides

Complete transverse blocks of each specimen were then embedded in paraffin and histological sections 3-4 microns thick were prepared.

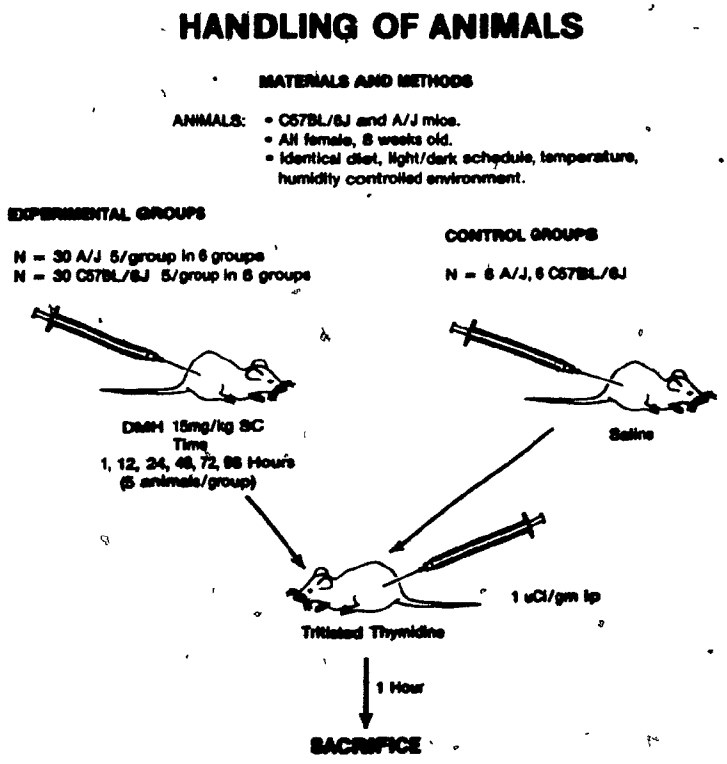

Figure 3. Schematic representation of materials and methods used in this experiment.

Autoradiographs were then processed by the dipping method of Leblond, Messier and Kapriwa (100). After a two week exposure time, slides were fixed and stained lightly with hematoxylin and eosin.

c) Crypt Analyses

In each autoradiograph a quantitative analysis was carried out using either the "left" or "right" sides of thirty perfect axial crypt sections. The criteria for selection being that the crypt be sectioned longitudinally with its base in contact with the muscularis mucosa and its mouth at the lumenal surface. Crypts were counted at random from the entire section of colon. The height of each crypt column was measured by counting the number of cells from base to mouth of the crypt. The number of labelled cells, and the position of each labelled cell was also recorded. In thirty additional crypts which were cut transversely to the lumen of the intestine, the number of columns was measured by counting the number of cells around the periphery of the crypt. Thus the number of cells/crypt column, multiplied by the number of columns/crypt gave the total number of cells/crypt for each colon scored.

Background "fog" was negligable and cells were considered labelled if seven or more grains were seen over the nucleus.

d) Distribution Analyses

In the control animals, distribution curves were derived by counting the percent of cells labelled at each cell position along the length of the crypt for each colon scored.

For the control groups, each animal had the mean number of cells per crypt column (i.e. crypt height), divided into thirds and each labelled cell was assigned to either the lower, middle or upper third

of the crypt for each colon scored.

e) Statistical Analysis

i) Control Animals

Means and standard deviations of all measurements were calculated for each strain. Differences in crypt column height, number of columns per crypt, number of cells per crypt, number of labelled cells per crypt column and LI were compared by applying the student t test. Strain differences in LI as a function of cell position within the crypt column were assessed by using a matched-pair t test.

ii) DMH-Exposed Animals

Differences between group means for crypt column height, LI and absolute number of labelled cells per crypt column across time in each DMH treatment group were calculated and their significance was tested using a mixed model analysis of variance.

(C) RESULTS

a) Control Animals

The mean body weight for the A mice was 21.5 ± 2.2 gm (mean ± SD) versus (vs) 17.2 ± 0.8 gm for the B mouse. Colon weights were 533.3 ± 37.2 mgm for A vs 388.3 ± 45.7 mgm for B. Colon lengths (cecum to anus were 14.0 ± .71 cm for A and 10.60 ± .29 cm for B. All of these values were significantly different ($p < .001$) (Table 3).

i) Proliferative Indices

As can be seen from Table 3, the proliferative indices for the A strain in the resting state were significantly greater than that for the B strain. The mean crypt height was 33.20 ± .75 cells for A and 28.80 ± .80 cells for B ($p < .01$). Also the mean number of labelled cells per crypt column (4.48 ± .56 vs 2.66 ± .86), the mean number of cells/crypt (524 ± 29 vs 430 ± 16) and the mean LI (13.40 ± 1.6 vs

Table 3

MORPHOMETRIC AND PROLIFERATIVE INDICES IN A/J AND C57BL/6J UNTREATED CONTROL MICE[a]

	C57BL/6J	A/J
Morphometric Indices:		
Body Weight (gms)	17.2 ± .8[b]	21.5 ± 2.2[d]
Colon Weight (mgm)	338.3 ± 45.7	553.3 ±37.2[d]
Colon Length (cm)	10.6 ± .3	14.0 ± .7[d]
Proliferative Indices:		
Column Height (cells)	28.80± .80	33.2 ± .75[e]
# Columns/Crypt	15.02± .21	15.70± .96
# Labelled Cells/Column	2.66± .86	4.48± .56[e]
# Cells/Crypt	430 ± 16[c]	524 ±29[e]
Labelling Index	9.1 ± 2.9	13.4 ± 1.6[e]

[a] Six animals per group

[b] Mean ± SD

[c] # Cells/crypt = column height x # columns/crypt

[d] $p < .001$ A vs B

[e] $p < .01$ A vs B

9.16 ± 2.9) were all significantly greater in the DMH-sensitive A strain (Figures 4-6). The only parameter that was not different was the number of columns/crypt (15.70 ± .96 vs 15.02 ± .21). This estimate had small inter-animal variance but wide inter-crypt variance, and depending on the level of cross section, i.e. closer toward the base vs closer toward the lumen, the crypt would have fewer or greater columns (Figure 7). This reflects the shape of the crypt and underscores the difficulty in deriving estimates of whole crypt populations in two dimensional analyses.

ii) Distribution Analysis

The LI for every cell position along the crypt was examined in both strains of mice (Figure 8). For all cell positions the DMH sensitive A strain has a higher percentage of cells labelled than the B strain. As well, the A mouse has labelled cells up to position 25 whereas the B strain has termination of DNA synthesizing cells at position 17. These two curves were compared by a matched-pair t test at each cell position and were significantly different ($p < .0001$; mean difference in labelling index = 6.40 ± 1.07%).

When dividing the crypt into thirds and assigning each labelled cell to either the upper, middle or lower third of the crypt, there was a total of 213 labelled cells in the A's middle third whereas the B's had a total of 90 labelled cells in this region. This represents approximately 26% of all A labelled cells vs 17% of B labelled cells. The upper third contained almost 1% of all labelled A strain cells vs none of the B strain cells (Table 4).

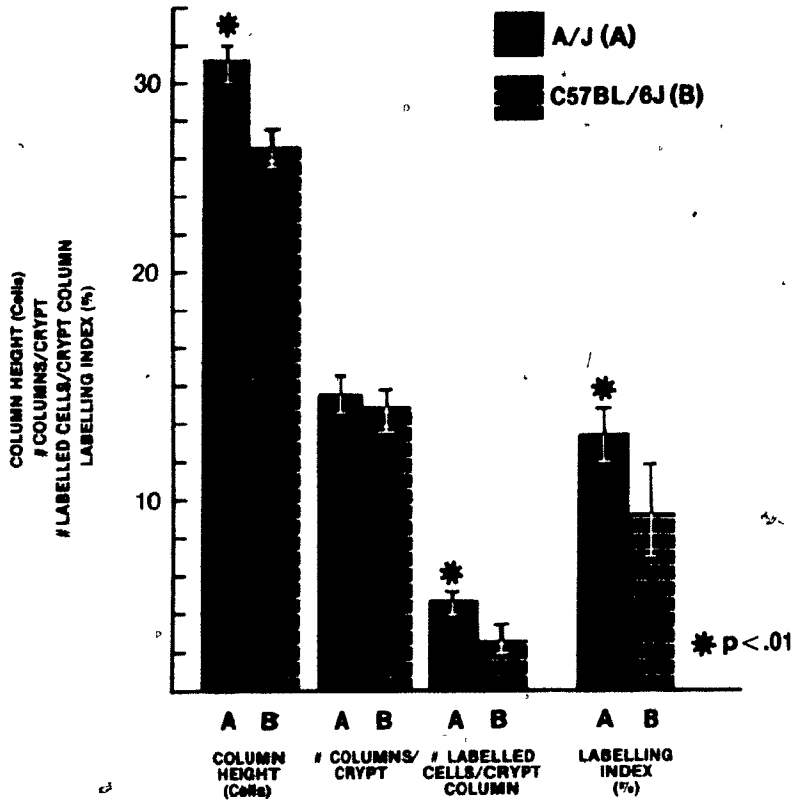

Figure 4. Proliferative parameters in untreated control A/J and C57BL/6J mice colonic crypts.

Figure 5. Autoradiograph of an untreated C57BL/6J mouse colonic crypt. Note the number and distribution of labelled cells.

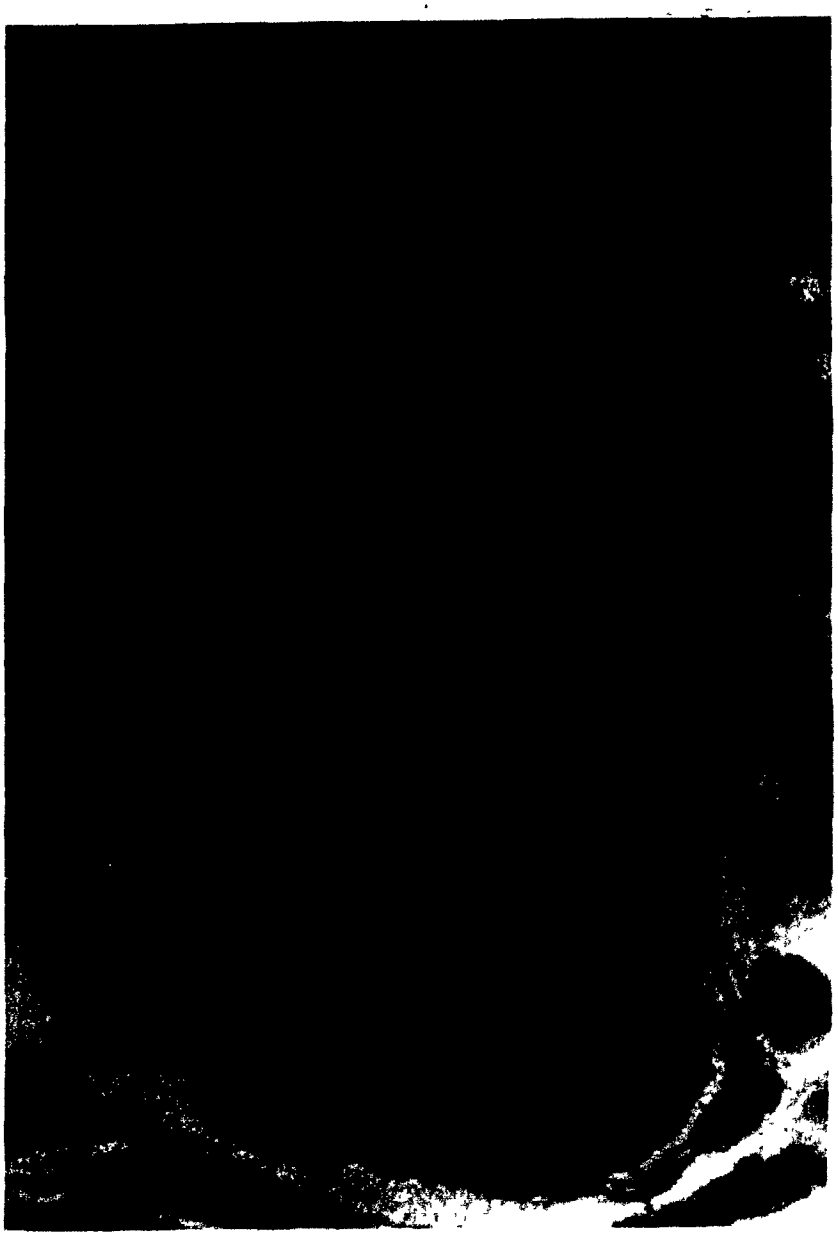

Figure 6. Autoradiograph of an untreated A/J mouse colonic crypt. Note the number and distribution of labelled cells.

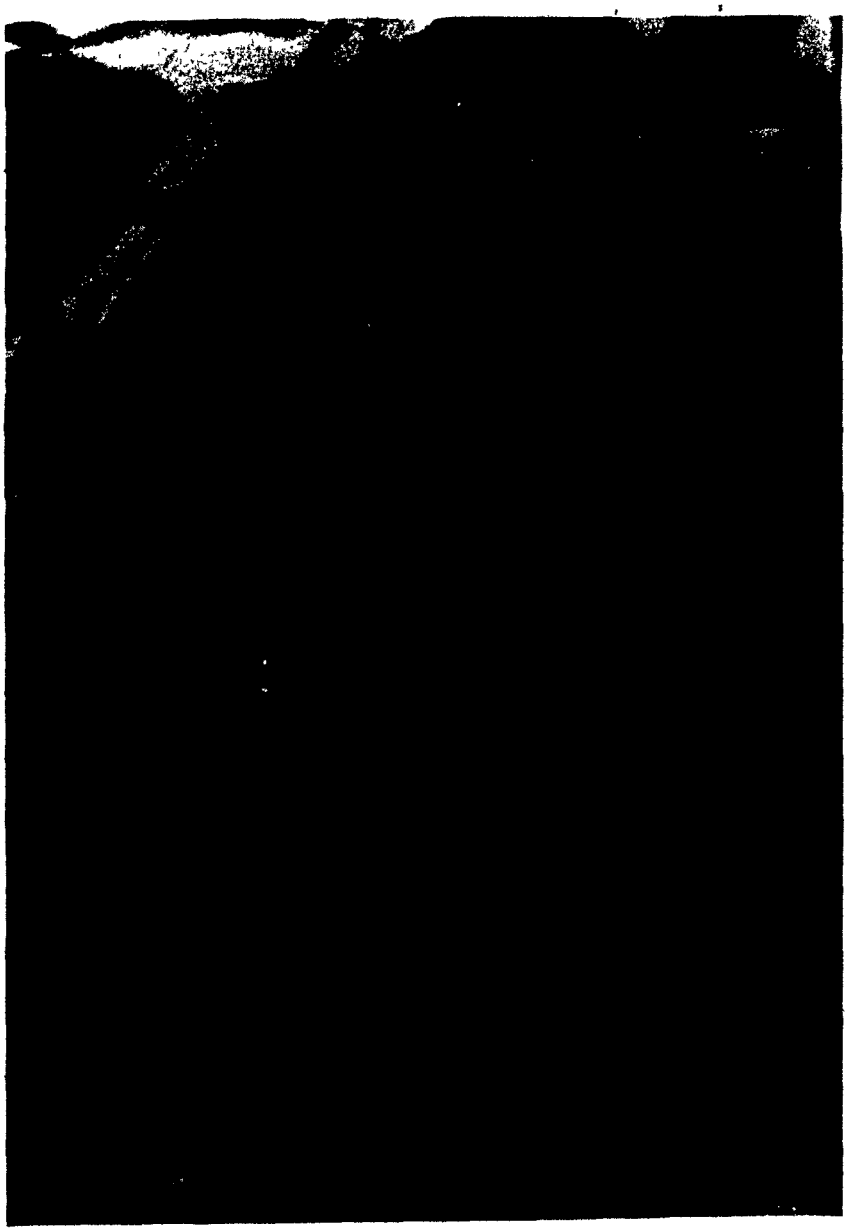

Figure 7. Autoradiographic cross-section of an A/J mouse colon. Note the variability in the number of crypt columns per crypt.

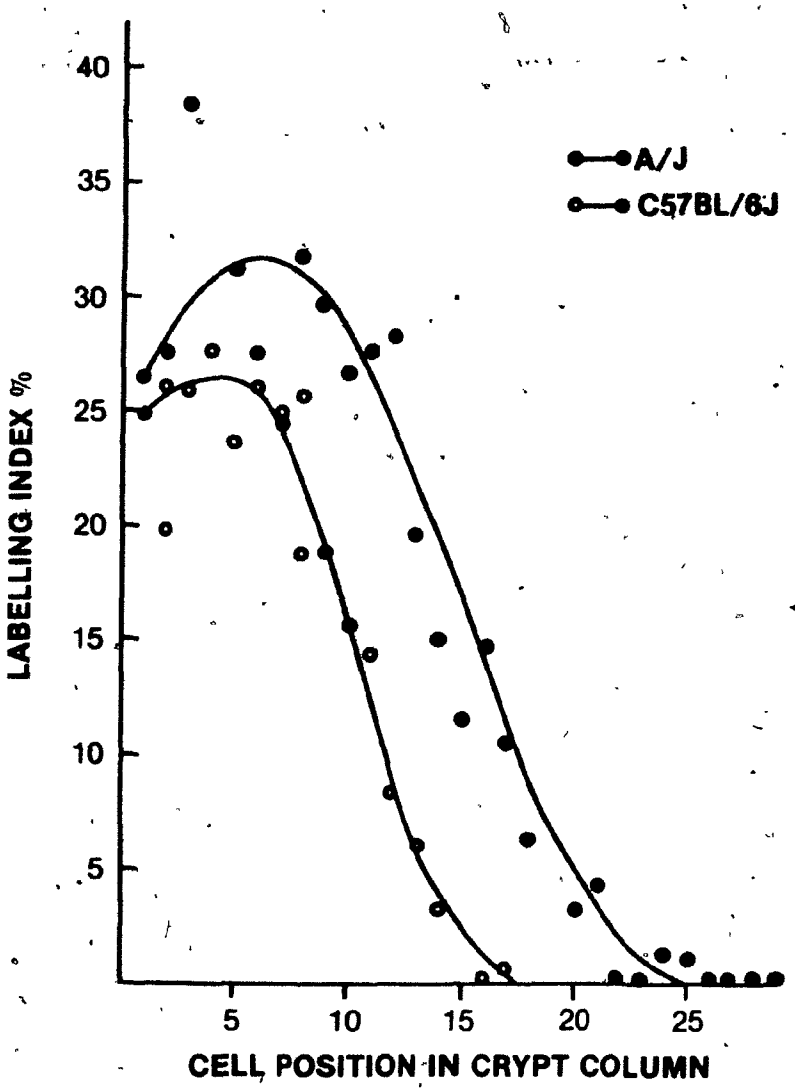

Figure 8. Labelling index for each cell position in crypt columns of untreated A/J and C57BL/6J mice. Each point represents the mean LI for each cell position scored in 6 animals of each strain.

Table 4

DISTRIBUTION OF LABELLED CELLS IN CRYPT COLUMNS OF A/J AND C57BL/6J UNTREATED CONTROL MICE[a]

Strain	Lower 1/3		Middle 1/3		Upper 1/3	
	# Labelled	%	# Labelled	%	# Labelled	%
A/J	577	73.5±8.6[b]	213	26.0± 7.6	6	.7±1.0
C57BL/6J	404	82.7±8.4	90	17.1± 8.4	0	0

[a]Values represent the mean percent or the absolute number of all labelled cells recorded in each third of the crypt for all animals scored. Each group contained 6 animals.

[b]Mean ± SD

b) DMH-Exposed Animals.

i) Weight Loss

The sensitive A animals lost a mean of 14% of their TBW 48 hours after DMH exposure (Figure 9) (% change in TBW = 1 - post-DMH; TBW/pre-DMH TBW x 100). The B strain lost a mean of 3.6% of TBW at 12 hours but gained this weight back within 24 hours. Subjectively the A strain were less active, ate less food, and had more diarrhea than the B strain.

ii) Proliferative Indices

As can be seen from Figure 10, at 12 hours both the A and B strains had a drop in the absolute number of labelled cells/crypt column, and a drop in the number of cells/crypt column. By 12 hours post-DMH, the A strain lost a mean of 7.1 cells or 21% of the column height and the B's lost a mean of 4 cells or 13.9% of column height. Both strains recovered by increasing the number of DNA synthesizing cells in their crypts and by 96 hours post-DMH the column heights, number of labelled cells and LI had all returned to pre-DMH levels. LI in both strains peaked at 48 hours, the A being 20.4 ± 1.4% and the B 15.9 ± 1.4% representing an increase in 1.52 times control for A and 1.75 times control values for B. As can be seen in Figure 10, this increase in the number of labelled cells per crypt column, coupled with the decrease in crypt column height results in a great increase in the LI in both the sensitive and resistant animals. The curves for crypt height, labelled cells/crypt column and LI were analyzed for each strain at each time interval by a mixed model analysis of variance. The curves for crypt column height are not statistically parallel probably because of the smaller difference at 48 hours post-DMH but we can still say that time effect is significant ($p < .0001$)

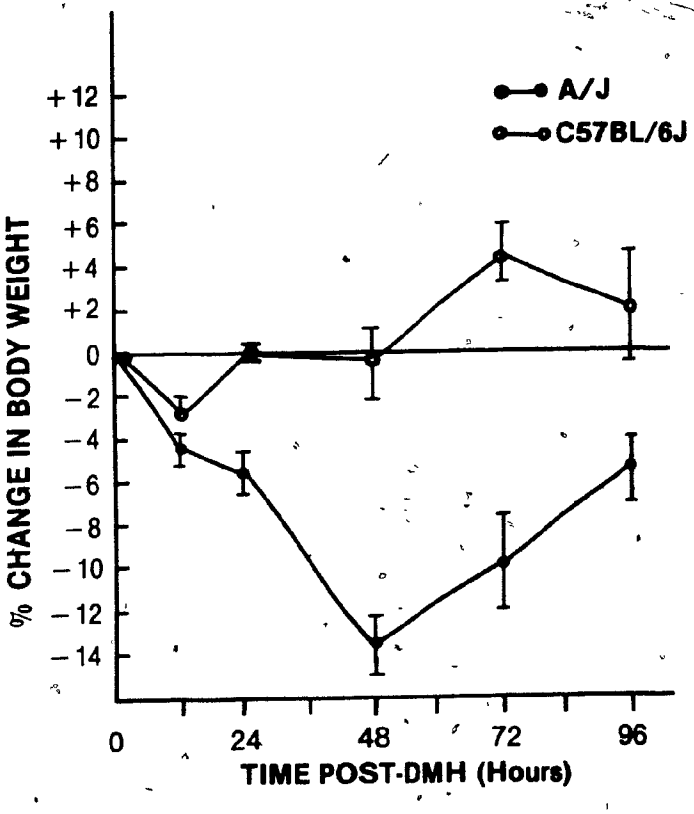

Figure 9. Percent change in the total body weight as a function of time following one exposure to DMH in A/J and C57BL/6J mice. Each point represents mean ± SEM for 5 animals.

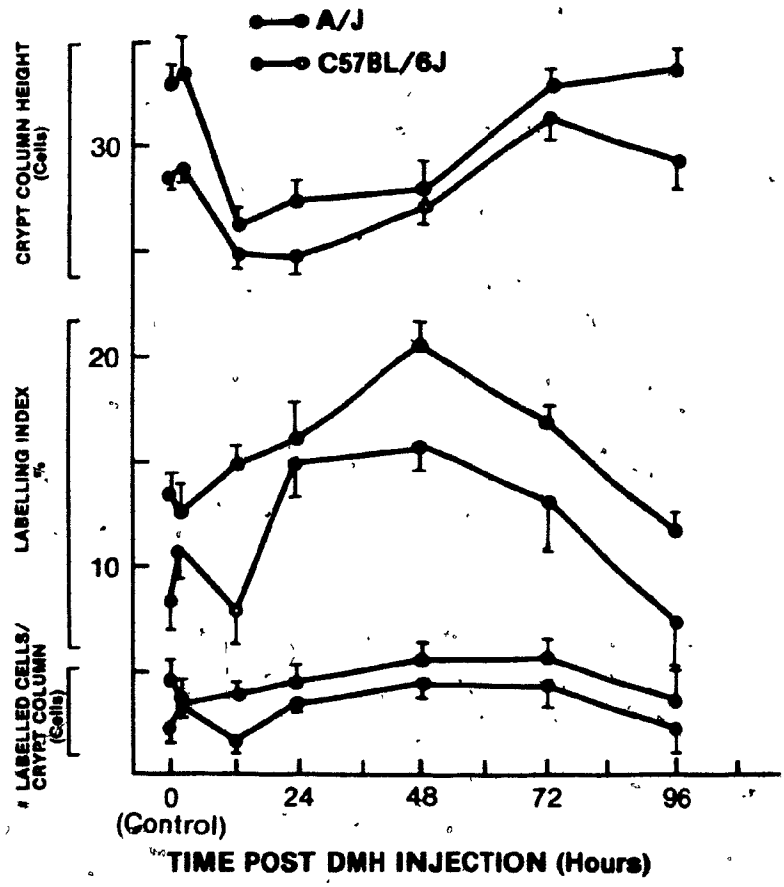

Figure 10. Changes in crypt height, labelling index and number of labelled cell/crypt column in A/J and C57BL/6J mice as a function of time following one exposure to DMH. Each point represents the mean ± SEM of 3-5 animals per time interval.

and that overall the A's had longer crypt columns than the B's ($p = .003$). The curves for LI are parallel and are significantly different over time ($p < .0001$), and the overall LI for A is greater than for B ($p = .003$). Finally, for the absolute number of labelled cells per crypt column, the curves are again parallel over time, and the A mice have an overall greater number of labelled cells per crypt column than the B mice ($p = .002$).

(D) DISCUSSION

The present results suggest that the sensitivity of a mouse to long-term DMH exposure is related to the indigenous proliferative characteristics of its distal colonic mucosa. The DMH sensitive A strain had a longer crypt column, more cells/crypt, more labelled cells/crypt column, a higher LI and a wider PC than the DMH resistant B strain.

In our laboratory, the A and B strains have been tested with chronic DMH exposure and the A's developed 19.0 ± 8.7 tumors/animals vs 1.0 ± 1.4 tumors/animal in the B's (Fleiszer, D.M., Skamene, E. - manuscript in preparation). This great difference in sensitivity to the carcinogen justified using these two inbred strains for analysis. Deschner has examined the proliferative characteristics of the C57BL/6J mice and found that they had a LI of 8.5 ± 1.1 (96). These findings are similar to ours.

In the DMH exposed groups of mice, the proliferative indices reflected the initial decrease and subsequent increase in numbers of DNA synthesizing cells. It is clear that the "overshoot" in the LI at 48 hours in both strains is due not only to the increase in numbers of labelled cells but also to the decrease in the crypt cell population itself. It has been shown that the early inhibition in DNA synthesis

is seen both in mice and rats (73-75) and this change reflects the cytotoxic effects of DMH, and the subsequent repopulation of the crypts. Our present study is in accordance with the work of others in demonstrating that the acute response to DMH has a specific pattern of cell loss and recovery (74, 75, 101). This response however was seen in both sensitive and resistant inbred strains, and therefore cannot explain the differences in eventual tumor formation after long-term exposure to the carcinogen.

Several studies have attempted to explain the different sensitivities to DMH based on differences in its metabolism (102, 103). The A strain lost a greater percentage of its TBW and appeared sicker than the B strain after exposure to DMH, but on the basis of changes in the proliferative indices following DMH exposure, the present study supports the concept that both inbred strains initially metabolize DMH in a similar fashion. It may be that the acute systemic effects are greater in the A strain, but the acute direct acting effects on the colon are of equal magnitude in both strains.

As suggested by Deschner (96), since A mice are initially as sensitive to DMH as B mice, the difference in eventual tumor load after chronic exposure to the carcinogen might be explained in terms of the transmission of stable mutations carried through several generations of DNA synthesis over time. Kanagalingam (104) proposed that the development of tumors results from the cell's inability to repair DNA damaged by chronic exposure to a carcinogen. In the A mouse, the resting LI is 50% greater and the absolute number of labelled cells is 70% greater than in the B mouse. Thus the greater number of proliferating cells in the colonic mucosa of the A strain

correlates with the degree of susceptibility to undergo neoplastic transformation. In the descending colon of the mouse, crypt cells have a short lifespan of about 100-130 hours and turnover rate of .75 - .95% cells/hour (105). In a renewing population like the colonic mucosa, errors in DNA could be incorporated into the next generation of cells if DNA repair is not carried out fully after exposure to a carcinogen. Thus errors could accumulate during chronic carcinogen exposure, and with a larger population of proliferating cells, such as in the A strain distal colon, more cells would be at risk, more errors would occur, and more tumors would subsequently develop.

The width of the PC has also been shown to correlate with DMH sensitivity (84, 96, 97). The A strain had 26.9% vs 17.1% in the B strain of its proliferating cells in the middle third of the crypt. These values support the concept that the wider the PC and the higher the labelled cells are on the crypt wall, the greater is the chance of transmitting a mutation after carcinogen exposure. It is of particular interest to note that almost 1% of all labelled cells in the sensitive A strain were in the upper third of the crypts. This pattern has been described as a stage I abnormality by Deschner (82, see Figure 2), where the PC extends beyond the middle third of the crypt. The stage I abnormality is usually seen after at least four injections of DMH in sensitive strains of mice (75, 77, 78, 81, 84, 96, 97). To my knowledge, no one has described the proliferative characteristics of the A/J mouse before. It would be of interest to follow the changes in the LI and PC of the A/J strain after chronic exposure to DMH.

The data in this study support the following conclusions: 1) inbred strains of mice have different proliferative characteristics in

their distal colonic mucosa; 2) the susceptibility to DMH-induced colon cancer can be predicted by the indigenous number and distribution of DNA synthesizing cells in the distal murine colonic mucosa; 3) the acute response in the proliferative compartment to DMH in different inbred strains of mice is similar and parallel; and 4) ultimate DMH carcinogenesis may depend on the establishment of stable transmissible mutations.

(E) SUMMARY

It has been proposed that the number and extent of tumors formed after chronic exposure to DMH can be predicted by the indigenous number and distribution of DNA synthesizing cells in the murine colonic mucosa, and that this sensitivity to DMH is genetically determined. In order to test this hypothesis, two genetically distinct inbred strains of mice, the DMH sensitive A mouse and the relatively DMH resistant B mouse were studied before and after a single exposure to DMH. The untreated A strain had a longer crypt column, a higher absolute number of labelled cells per crypt column, a greater LI, a wider PC, and a greater number and percentage of labelled cells in the middle and upper third of the crypt than the untreated B strain. After acute exposure to DMH, the A strain lost more of its TBW than the B strain. There was an initial loss of cryptal cells, a drop in the LI, and a subsequent increase and overshoot in the number of labelled cells and LI. This pattern of cell loss and recovery over time was parallel in both strains, and thus cannot explain the differences in ultimate tumor formation after chronic exposure to the carcinogen. The data are consistent with the theory that the susceptibility to DMH carcinogenesis can be predicted

by the indigenous proliferative characteristics of the murine colonic mucosa. The acute proliferative response to DMH in these strains is similar and parallel, thus ultimate tumor load may depend on long-term effects such as the establishment of stable transmissible mutations.

EXPERIMENT II

1,2-DIMETHYLHYDRAZINE INDUCED NUCLEAR ABERRATIONS IN C57BL/6J AND A/J MOUSE COLONIC CRYPTS

9. 1,2-DIMETHYLHYDRAZINE INDUCED NUCLEAR ABERRATIONS IN C57BL/6J AND A/J COLONIC CRYPTS

(A) INTRODUCTION

In recent years there has been a great deal of interest in developing specific and reliable markers for colonic carcinogen exposure. DMH is a carcinogen that after long-term exposure reliably induces tumors in the distal murine colon (22). Although the precise mechanism by which DMH induces colon tumors is not completely understood, it has been shown to methylate DNA of cells in its target tissues (106-108), to inhibit normal DNA synthesis (109, 110) and to cause degenerative changes in the cells lining the colonic crypts of the distal murine colon (23, 81, 89-91). DMH is site-specific for the distal colonic mucosa and it is this region where the greatest cytotoxic and proliferative changes occur, as well as the greatest tumor load following chronic DMH exposure (74, 110-112).

Most studies have examined tumor load as the end-point of DMH carcinogenesis but several recent reports have examined the acute cytotoxic effects of DMH in the colon as a measure of exposure to the carcinogen (75, 87, 89-91, 95, 113). These measurements are based on the detection and quantification of nuclear aberrations that appear in the crypt epithelium before and after treatment with a known carcinogen. Maskens (91, 111) demonstrated that the proportion of karyorrhectic nuclei following carcinogen exposure correlates well with a drop in the mitotic index and with the sensitivity of individual segments in the rat colon to eventual tumor development. Heddle et al (87) used the micronucleus test as a means of characterizing and quantifying carcinogen exposure. Wargovich et al (75) measured several forms of nuclear anomalies and demonstrated that

their numbers had a specific pattern as a function of time following DMH exposure.

The quantification of colonic nuclear aberrations as a measure of exposure to a colon carcinogen appears to reliably discriminate tissue specific carcinogens from non-carcinogens. Non-colon carcinogenic analogues of DMH such as 1,1-dimethylhydrazine or n-nitrosodimethylamine do not increase the number of micronuclei after their administration (87, 90). Thus the quantification of nuclear aberrations is not only a sensitive assay for carcinogen exposure, but a specific one as well.

In this study in order to facilitate the quantification of DMH-induced cellular damage, all forms of nuclear abnormalities, that is, pyknotic, karyorrhectic and micronuclei have been grouped under one heading, nuclear aberrations (NA). Thus all NA are considered as a part of the continuum from cell damage, to cell death, to eventual cell extrusion or digestion.

In this study the absolute and relative numbers of NA in the distal 3 cm of colonic mucosa in two different inbred strains of mice before and after exposure to DMH are examined. The strains are the DMH sensitive (tumor forming) A mouse and the relatively DMH resistant (non-tumor forming) B mouse. The colonic crypts are also scored for numbers of mitotic figures (MF) and for crypt column height.

The purpose of this study is to 1) quantitate the absolute and relative number of aberrant nuclei and mitotic figures in the distal colonic crypts of two different inbred mouse strains as a function of time following a single DMH exposure; 2) compare the nuclear aberration index (NAI) and the mitotic index (MI) in these mouse

strains as a function of time following DMH exposure; 3) evaluate the NAI assay as an indicator of acute DMH exposure, and finally to 4) evaluate the usefulness of the NAI assay as a predictor of ultimate neoplastic growth potential.

(B) MATERIALS AND METHODS

a) Animals

A and B mice were obtained from the Jackson Lab (Bar Harbor, Maine). All animals were female and 7 weeks old at the time of purchase, and 8 weeks old at the start of the experiment. All mice were housed 4-5 per cage on wood chip bedding, in a 12 hour light/dark cycle, temperature and humidity controlled environment. Animals were allowed water and mouse chow (Ralston Purina Co., St. Louis, Missouri) ad libitum.

b) Carcinogen Treatment

Fresh solutions of DMH (Aldrich Chemical Co., Milwaukee, Wisconsin) were made by dissolving 150 mg of DMH in 50 ml of NaCl and pH was adjusted to 6.5 with NaOH. Each experimental mouse received 15 mg/kg DMH subcutaneously once only. DMH was given to 25 mice of each strain and groups of 5 animals were sacrificed at 12, 24, 48, 72 and 96 hours following treatment. All animals were sacrificed between 10 and 11 a.m. to avoid diurnal fluctuations in proliferative and apoptotic parameters (59, 60, 114). Five untreated animals of each strain served as controls and were sacrificed at time 0.

c) Tissue Preparation

All animals were killed by cervical dislocation and their colons were excised from the abdominal cavity, and flushed with cold NaCl solution. The colon was slit open from anus to cecum and pinned mucosal surface upward on a cork board and fixed in 10% buffered

formaldehyde for 24 hours. Histologic sections (3-4 microns thick) of the distal 3 cm of colon were cut from paraffin embedded blocks and slides were stained with feulgen and a fast green counterstain.

d) Crypt Analyses

The position and number of all NA and MF were scored in 20 crypt sections for each animal. Only crypts that were sectioned along their long axis extending from the muscularis mucosa to crypt mouth were scored. Crypt column height was determined by counting the number of cells along the "right" or "left" sides of 30 crypt columns for each colon scored.

The distribution of NA within the crypt was determined by scoring aberrations for each cell position where position 1 represents the bottom cell of the crypt section. The NAI in the distribution curve is determined by pooling the total number of NA for each animal at each cell position and dividing by the total number of cells counted.

Thus the distribution NAI =

$$\frac{\text{total number of nuclear aberrations per cell position}}{\text{total number of cells per position}} \times 100$$

For determining the change in frequency of NA and MF as a function of time after DMH exposure, means and standard deviations of the absolute numbers of NA and MF per crypt section were calculated for each group of animals (4-5 per group). For determining the change in frequency of the NAI and MI as a function of time, the absolute number of NA and MF were divided by 2 and then divided by the mean crypt height (in cells) for each animal. Thus in this instance, NAI and MI represent the percentage of aberrant or mitotic nuclei in the entire crypt column.

e) Statistical Analysis

Frequencies of all NA and MF for each treatment group were compared to untreated controls by the student t test. Values were considered significant if $p < .01$. NAI and MI values were similarly compared. Correlation between crypt column heights and NA at each time interval were determined using a simple linear regression curve.

(C) RESULTS

The A and B untreated control mice had similar baseline numbers of NA and their distributions were also similar (Figures 11 and 12). DMH induced all three types of NA in both strains of mice. In the 12 hour groups, the NAI at each cell position in both strains was similar with the greatest percentage of aberrant cells between cell positions 1 and 10 (Figure 11). Up to 50% of B cells and 40% of A cells at position 3 were aberrant 12 hours after DMH treatment. Distribution curves for the 24, 48, 72 and 96 hour groups are not presented here, but the patterns were similar for both strains of mice. All NA appeared in the lower two thirds of the crypt sections, and by 96 hours, the curves were indistinguishable from the untreated control curve. Although no quantitative discrimination was made between the types of NA, in the control animals most of the aberrations were in the form of micronuclei. This is in contrast to the DMH treated animals where karyorrhectic and pyknotic nuclei appeared in greater numbers (Figure 13).

The frequency of NA and MF per crypt section following DMH as a function of time is seen in Figure 14. The greatest increase in NA occurred between 12-24 hours post-DMH in both strains. This is followed by a gradual decrease in numbers of NA to pretreatment levels

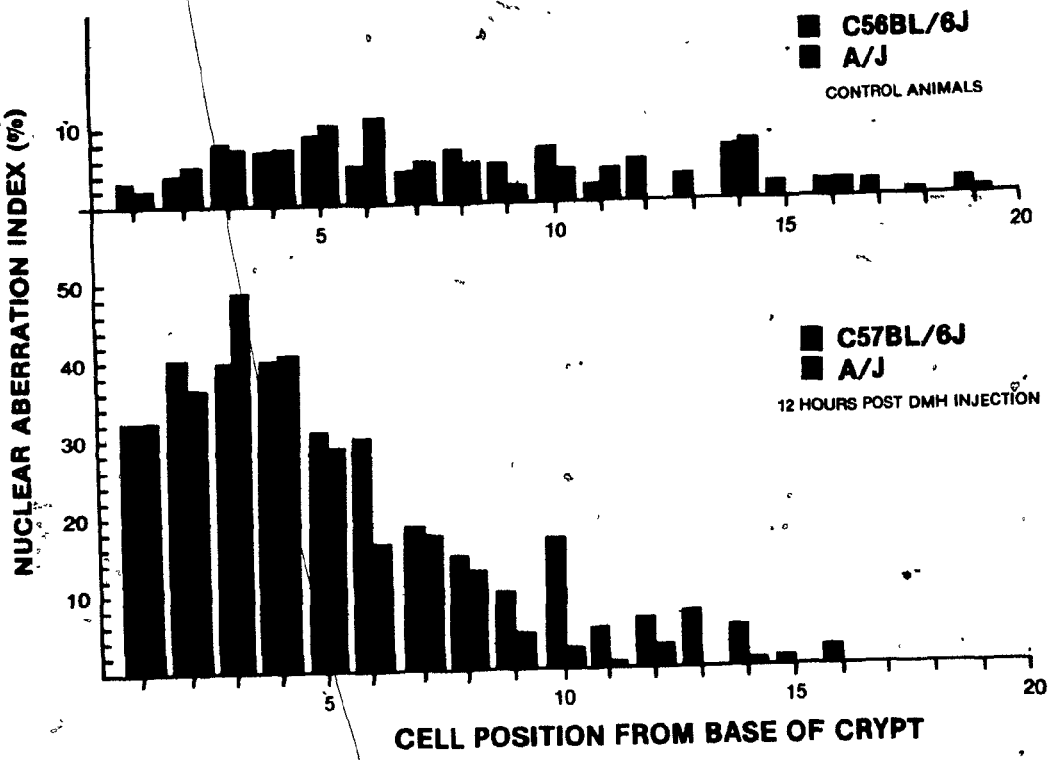

Figure 11. Distribution of nuclear aberrations in C57BL/6J and A/J mice within the crypt section as a function of cell position. Upper panel = untreated controls; lower panel = 12 hours post-DMH exposure.

Figure 12. Photomicrograph of an untreated C57BL/6J mouse colonic crypt stained with feulgen and fast green counterstain. Arrow indicates single micronucleus at base of crypt.

Figure 13. Photomicrograph of a C57BL/6J mouse colonic crypt 24 hours post-DMH stained with feulgen and fast green counterstain. Arrows indicate multiple nuclear aberrations in lower third of crypt.

by 96 hours. The A controls had .89 ± .19 (mean ± S.D.) NA per crypt section and this rose to 3.13 ± .49 at 12 hours post-DMH. This represents a 3.5-fold increase. Similarly, the untreated B mice had a baseline of .7 ± .2 NA per crypt and 2.38 ± .31 at 12 hours; also a 3.5-fold increase. Both these values are significantly greater than the control values ($p < .001$).

The number of MF scored for each strain as a function of time post-DMH was also determined (Figure 14). The patterns are similar for both strains and show an initial suppression and subsequent increase and overshoot in the MF rate. The untreated A and B mice had .43 ± .16 and .55 ± .37 MF per crypt section, respectively (Figure 15), and by 48 hours after DMH treatment, this rose to 1.08 ± .20 and 1.07 ± .17 MF per crypt (Figure 16). This represents a 2.5 and 2.0-fold increase for the A and B mice, respectively. These values are significantly different than the control values ($p < .01$).

Because there are interstrain differences in crypt height and thus crypt cell population (71, 115), we again scored the frequency of NA and MF, but this time as a percentage of the total number of cells in the crypt column. Thus crypt column heights were determined for each animal scored (Table 5) and the NAI and MI were determined as a function of time after DMH exposure.

Figure 17 represents the NAI and MI of each strain after DMH treatment. The mean NAI in untreated A and B mice were 1.34 ± .28% and 1.20 ± .36%, respectively. These values rose to 5.8 ± .56% and 4.79 ± .76% by 12 hours. This represents a 4-fold increase in both strains.

As seen in Table 5, the temporal changes in crypt column height correspond to the increase in NA. Thus the loss in crypt column

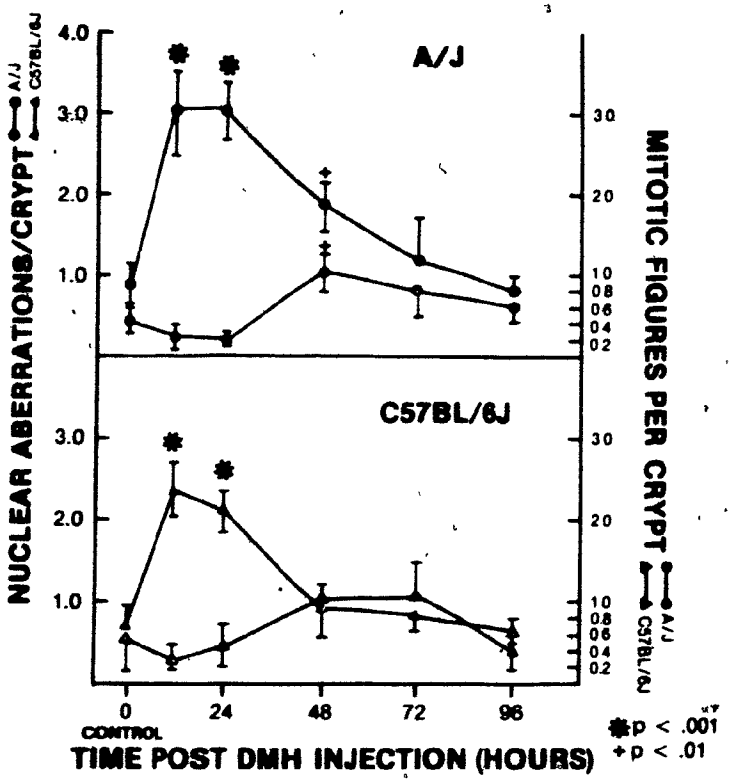

Figure 14. Frequency of nuclear aberrations and mitotic figures as a function of time following one DMH exposure in C57BL/6J and A/J mice. Each point represents the mean ± SD of 4-5 animals.

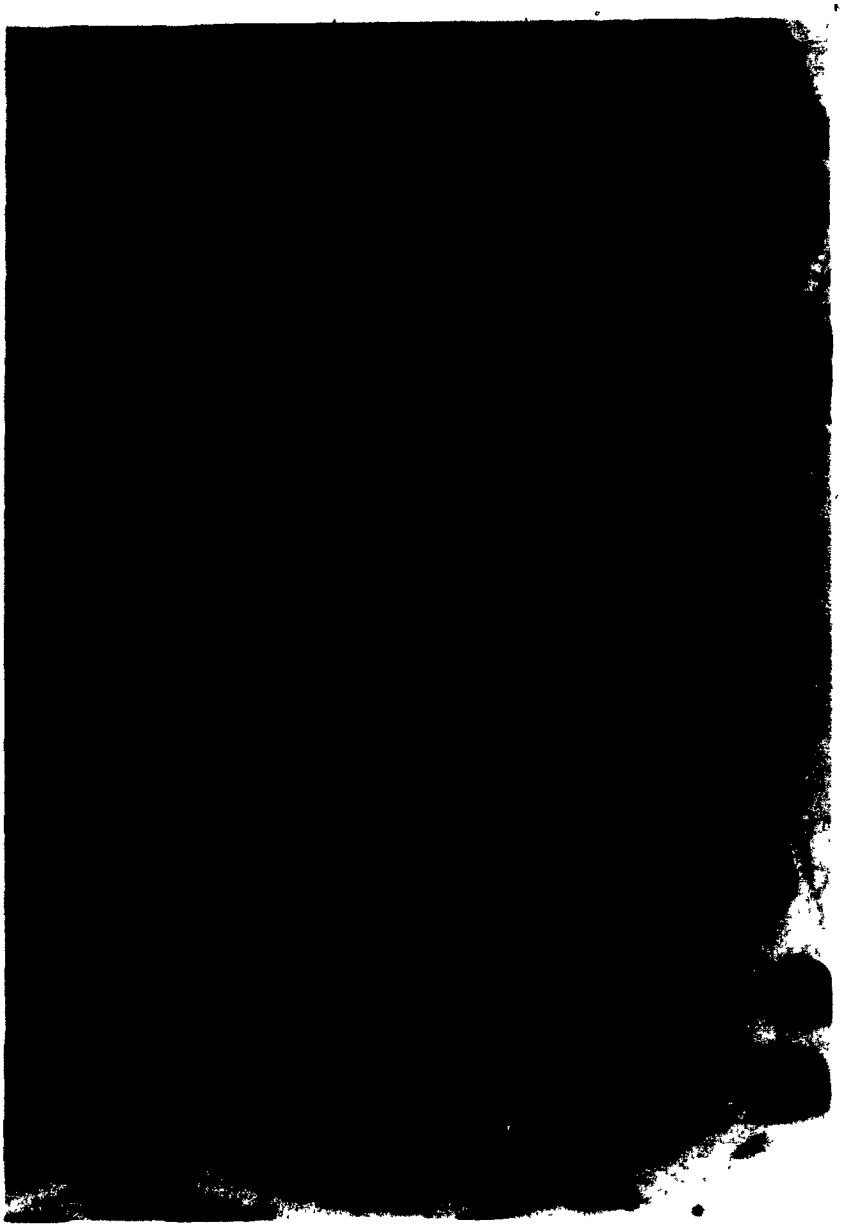

Figure 15. Photomicrograph of an untreated A/J mouse colonic crypt stained with feulgen and fast green counterstain. Arrow indicates single mitotic figure.

Figure 16. Photomicrograph of an A/J mouse colonic crypt 48 hours post-DMH stained with feulgen and fast green counterstain. Arrows indicate multiple mitotic figures.

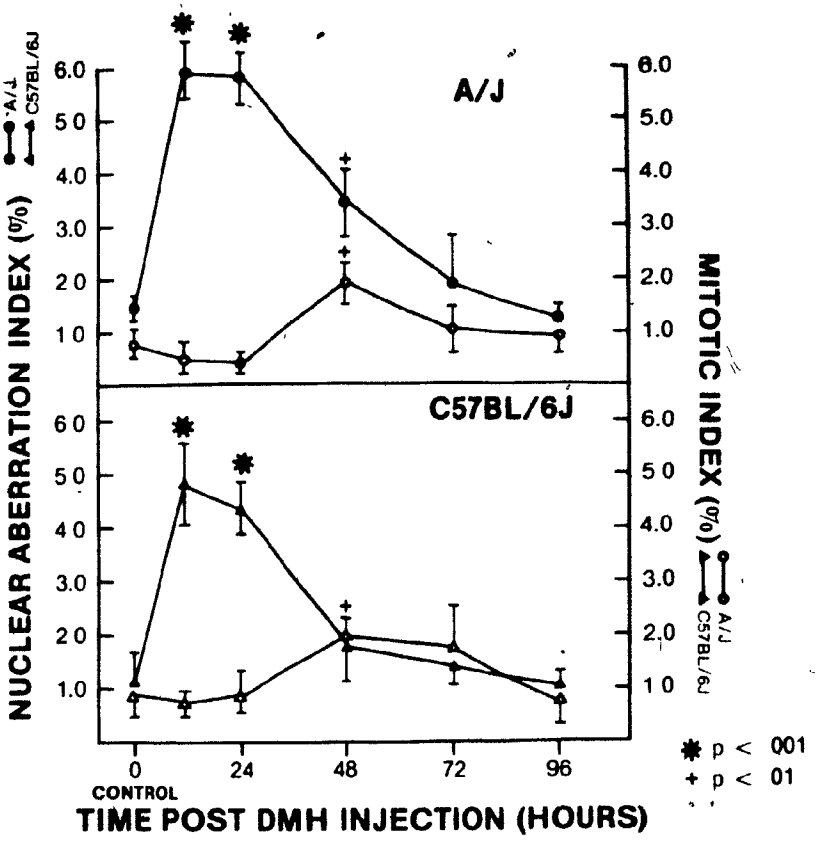

Figure 17. Nuclear aberration index and mitotic index in A/J and C57BL/6J mice as a function of time following one DMH exposure. Each point represents the mean ± SD (in percent) of all cells scored for each group of mice.

Table 5

CHANGE IN CRYPT COLUMN HEIGHT AS A FUNCTION OF TIME AFTER DMH EXPOSURE

Strain	Time Post-DMH ~~Injection~~					
	0	12	24	48	72	96
A/J	33.2±.75[b]	26.9±1.70[c]	27.1±1.80[c]	27.0± .54[c]	32.7±.65	33.3± .47
C57BL/6J	28.8±.79	24.9± .79[c]	24.8± .68[c]	27.5±2.10	31.1±.90	29.2±1.0

[a] DMH 15 mg/kg sc x 1 dose. 30 crypt columns scored (in cells) for each animal, 4-5 animals per time period.

[b] mean ± S.D.

[c] $p < .01$ when compared to controls (time 0)

height is due to the loss of viable cells in the column which are degenerating. The correlation coefficient for NA vs column height for the A mice is -.84, and for the B mice r = -.86.

(D) DISCUSSION

It has been suggested that the measurment of nuclear aberrations in the colon crypt is an in vivo short-term assay that is a reliable, sensitive and specific method of screening for exposure to a colon carcinogen (87, 89-91). In this study both the absolute and relative numbers of NA, MF and crypt column height were quantified as a function of time following a single exposure to the colon carcinogen DMH. Untreated animals in both strains had similar baseline aberration patterns. Induction of NA was found to peak at 12-24 hours after DMH and return to pretreatment levels by 96 hours in both A and B mice. These findings are in agreement with other reports (75, 89, 91).

The distribution of NA at 12 hours post-DMH reveals that the vast majority of nuclear damage occurs in the bottom two thirds of the crypt. This is the proliferative compartment of the crypt and cells in this region have the greatest synthesis of DNA and highest labelling indices (84). The distribution pattern reported here is in agreement with other series previously reported (75, 87, 91, 96, 97, 113). It is worth noting that the 12 hour NAI distribution curve (Figure 11) closely resembles the labelling index distribution curve (68, 71, 96, 97; see Figure 8). It has been suggested that proliferative activity must be a prerequisite for carcinogen-induced apoptosis (89). Thus as an alkylating agent, DMH primarily damages the nuclear DNA and this results in the formation of aberrant nuclei

that appear within the proliferative compartments of the colonic crypts.

In this study both the absolute and relative frequencies of NA and MF were quantified. The NAI is a more precise index of carcinogen exposure because it takes into account the total number of cells at risk. This distinction is a critical one because the A mice were found to have a baseline crypt column height of 33.2 ± .7 cells, vs 28.8 ± .7 cells in the B mice ($p < .01$). Thus the A mice had a greater number of cells at risk for carcinogen exposure. In fact, A mice have a significantly higher labelling index and a wider proliferative compartment than the B mice (115). Both the labelling index and width of the proliferative compartment are thought to relate to and be predictive of the sensitivity of a given mouse strain to long-term DMH carcinogenesis (96, 97). This is an important concept in light of the similar patterns of DMH-induced nuclear damage in the tumor forming A and relatively non-tumor forming B mice. The initial suppression of DNA synthesis, the increase in frequency of aberrant nuclei and loss of crypt column height are all measures of DMH toxicity and are similar and parallel in both strains of mice. Thus the A and B mice most likely initially metabolize DMH to an equal degree. This argues against the theory that ultimate tumor load depends principally on a difference in the metabolism of the carcinogen (102, 103). However, these similar patterns cannot explain why the A mouse develops 19 ± 8.7 tumors per animal while the B mouse develops 1.0 ± 1.4 tumors per animal following 20 weekly injections of DMH ($p < .01$) (Fleiszer, D.M. and Skamene, E., manuscript in preparation). Therefore DMH induces NA in both strains of mice, but the NAI assay does not relate to eventual neoplastic transformation.

It should not be used as a predictor of a given strain's ultimate sensitivity to a carcinogen. The NAI assay's greatest strength lies in its potential as an indicator of early carcinogen exposure.

Thus the data presented here indirectly supports the theory that tumorogenic potential may depend on the proliferative parameters of colonic crypt cells, and the establishment of stable mutations carried through several generations of cell division over time. Since the initial effects of DMH are similar in different inbred strains of mice that have known dissimilar sensitivities to long-term DMH exposure, the later processes involved in either protection of promotion of carcinogenesis should be areas for future investigation. These modifiers of carcinogenesis might include various immune parameters, humoral responses, oncogenes or DNA repair mechanisms.

(E) SUMMARY

A single exposure to DMH produces several forms of aberrant nuclei in the crypts of the distal murine colon. The frequency of nuclear aberrations in the distal colonic crypts in DMH-sensitive A and relatively DMH-resistant B mice before and after a single exposure to DMH were examined. Nuclear aberrations, mitotic figures and crypt column heights were scored for all animals as a function of time following administration of DMH. In both strains there is a significant increase in the absolute and relative frequency of nuclear aberrations by 12 hours, with a corresponding drop and subsequent overshoot in the mitotic index by 48 hours post-DMH. The temporal changes in crypt column height correlate closely with the temporal changes in frequency of nuclear aberrations in both strains. The results suggest that both inbred strains respond to acute DMH exposure

in a similar and parallel fashion over time. The following conclusions can be drawn from these results: 1) that the nuclear aberration index assay is a sensitive method for detecting early DMH exposure and 2) that this assay does not relate to ultimate outcome after chronic DMH exposure, and therefore should not be used as a predictor of eventual neoplastic transformation.

PART III

10. FINAL SUMMARY AND CONCLUSIONS

Colon cancer is among the most prevalent malignancies in North America and other westernized countries. Migration, correlation and comparison studies have shed much light on the etiological roles of diet and fecal factors in colonic carcinogenesis. Animal experiments using the DMH model of colonic carcinogenesis have made significant contributions towards separating out the complex and interacting endogenous and exogenous etiologic factors. DMH has several features which make it extremely well suited for the systematic examination of these risk factors. Firstly, DMH colon carcinogenesis is site-specific for the distal colon and secondly, the tumors that develop are pathologically analagous to human colonic neoplasms.

Both human and animal studies have demonstrated that diets low in fiber and high in fat and protein are potentially promotive in their effects on colonic carcinogenesis. Although diet and environmental factors are still considered the major determinants of colon cancer, genetic predisposition plays a significant role in its genesis, both in the human and the animal models. Just as in so many disease systems, the cause of colon cancer remains unknown but must involve the interaction of host and environmental factors. Thus although the contribution of individual genetic predisposition is poorly defined, it could play a significant role in the pathogenesis of colon cancer, no matter how definitive the contribution of environmental factors.

Genetic predisposition to colon cancer may involve many pathways leading to diverse phenotypic changes. One of these pathways, cellular proliferation, has been studied extensively both in man and in the rodent. It has been suggested that cell proliferation might be used as a biological marker for human colonic neoplasia.

In the normal state proliferation is confined to the lower two thirds of the colonic crypts with the basal third the major zone of DNA synthesis. Deviations in the normal patterns of cell renewal have been characterized both in human and animal colon specimens. These alterations include an extension of the proliferative compartment towards the surface epithelium, an overall increase in the labelling index and a shift of the major zone of cell proliferation from the base to the middle and upper thirds of the glands. Again, this sequence of proliferative abnormalities has been described both in man and rodent and the pattern that emerges suggests that colonic crypt cell kinetics are closely associated with neoplastic growth potential.

In the present study, the colonic crypt cell proliferative characteristics and carcinogen induced nuclear aberrations of two strains of mice with known different sensitivities to DMH carcinogenesis were examined. The sensitive strain was found to have a longer crypt column, a higher labelling index, a wider proliferative compartment and a greater percentage of its DNA synthesizing cells in the upper two thirds of its crypts than the resistant strain; yet acutely, both strains responded to DMH in a similar and parallel fashion. The current theories of cellular kinetics and their relationship to neoplastic growth potential are clearly supported by the data and conclusions drawn in this study. The distinct kinetic patterns in these two genetically homogeneous mouse strains appear to reliably predict each strain's susceptibility to DMH-induced colon tumors.

The nuclear aberration index assay has only recently been described and is refuted to discriminate colon carcinogens from non-

colon carcinogens. The present study does not assess this reported specificity, but the data does demonstrate that nuclear aberrations are indeed induced in the crypts after only one exposure to DMH. The distribution of these aberrant nuclei supports the concept that the cells in the proliferative compartment are the ones most sensitive to carcinogeneic induced mutations. Again, both strains exhibited nuclear aberrations of equal magnitude and in similar distributions suggesting that other mechanisms, perhaps DNA repair systems or immune parameters are responsible for the long-term differences in tumor induction.

The ultimate goal of the gastrointestinal or surgical oncologist who is interested in colon cancer, is the early detection, prevention and cure of this disease. Although great strides have been made in the last thirty years in characterizing the etiological factors and the biological behavior of these tumors, little or no improvement in survival has resulted. The overall five year mortality rate still remains close to 40%, and this statistic has not changed in over a decade.

Certainly research must continue in the areas of treatment and cure of established disease. However, by formulating a screening technique based on simple cytokinetic measurements, the goal of preneoplastic detection and early identification of individuals or populations at increased risk for developing colon cancer might be realized.

11. BIBLIOGRAPHY

1) Wynder E.L. The epidemiology of large bowel cancer. Cancer Res. 35:3388, 1975.

2) Lambert R. Epidemiology of Colorectal Carcinogenesis. In: Colonic Carcinogenesis, Malt R.A., Williamson R.C.N. (eds). Falk Symposium 31, MTP Press, p. 1-10, 1982.

3) Jenson O.M. Colon Cancer Epidemiology. In: Experimental Colon Carcinogenesis, Autrup H., Williams G.M. (eds). CRC Press, New York, p. 3-23, 1983.

4) Cancer Facts and Figures. New York, American Cancer Society, Inc., 1984.

5) Cancer Statistics. CA 35:19, 1985.

6) Haenszel W. Cancer mortality among the foreign born in the U.S. JNCI 26:37, 1961.

7) Kmet J. The role of migrant populations in studies of selected cancer sites: A review. J. Chronic Dis. 23:305, 1970.

8) Gregor O., Toma R. and Prasova F. Gastrointestinal cancer and nutrition. GUT 10:1031, 1969.

9) Armstrong B. and Doll R. Environmental factors in cancer incidence and mortality in different countries with special reference to dietary practices. Int. J. Cancer 15:617, 1975.

10) Weisburger J.H. Mechanisms of action of diet as a carcinogen. Cancer 43:1987, 1979.

11) Burkitt D.P. Epidemiology of cancer of the colon and rectum. Cancer 28:3, 1971.

12) Graham S.M. and Mettlin C. Diet and colon cancer. Am. J. Epid. 109:1, 1979.

13) Burkitt D.P., Walker A.R.P. and Painter N.S. Effect of dietary

fibre in stools and transit times and its role in the causation of disease. Lancet 2:1408, 1972.

14) Freeman H.J. Dietary Fibers and Colonic Cancer. In: Experimental Colon Carcinogenesis, Autrup H., Williams G.M. (eds). CRC Press, p. 267-279, 1983.

15) Aries V., Crowther J.S., Draser B.S., Hill M.J. and Williams R.E.O. Bacteria and the aetiology of cancer in the large bowel. GUT 10:334, 1969.

16) Hill M.S., Draser B.S. and Aries V. Bacteria and the etiology of cancer of the large bowel. Lancet 1:95, 1971.

17) Hill M.J. The aetiology of colon cancer. Crit. Rev. Toxicol. 4:31, 1975.

18) Murray, W.R., Blackwood A., Trotter J.M., Calman K.C. and Mackay C. Fecal bile acids and clostridia in the aetiology of colorectal cancer. Br. J. Cancer 41:923, 1980.

19) Finegold S.M., Atteberg H.R. and Sutter V.V. Effect of diet on human fecal flora: Comparison of Japanese and American diets. Am. J. Clin. Nutr. 27:1456, 1974.

20) Thompson M.H. The Role of Fecal Microflora in Colon Carcinogenesis. In: Experimental Colon Carcinogenesis, Autrup H., Williams G.M. (eds). CRC Press, p. 253-266, 1983.

21) Druckrey H. Production of Colon Carcinomas by 1,2-Dialkylhydrazine and Azolyalkanes. In: Carcinoma of the Colon and Antecedant Epithelium, Burdette W.J. (ed). Charles C. Thomas, Springfield, p. 267-299, 1970.

22) Thurnherr N., Deschner E.E., Stonehill E.H. and Lipkin M. Induction of adenocarcinomas of the colon in mice by weekly

injections of 1,2-dimethylhydrazine. Cancer Res. 33:940, 1973.

23) Pozharisski K.M., Likhachev A.J., Klimashevski V.F. and Shaposhnikvo J.D. Experimental intestinal cancer research with special reference to human pathology. Adv. Cancer Res. 30:165, 1979.

24) Reddy B.S., Narisawa T., Moronpot R., Weisburger J.H. and Wynder E.L. Animal models for the study of dietary factors and cancer of the large bowel. Cancer Res. 35:3421, 1975.

25) Fleiszer D.M., MacFarlane J., Murray D. and Brown R.A. Protective effect of dietary fibre against chemically induced large bowel tumors in rats. Lancet ii:503, 1978.

26) Trudel J.L., Senterman M.K. and Brown R.A. The fat/fibre antagonism in experimental colon carcinogenesis. Surgery 94:691. 1984.

27) Nigro N.D., Singh O.V., Campbell R.L. and Pak M.S. Effect of dietary beef fat on intestinal tumor formation by azoxymethane in rats. JNCI 54:429, 1975.

28) Reddy B.S., Narisawa T. and Weisburger J.H. Effect of a diet with high levels of protein and fat on colon carcinogenesis in F344 rats treated with 1,2-dimethylhydrazine. JNCI 57:567, 1976.

29) Reddy B.S. Dietary fat and its relationship to large bowel cancer. Cancer Res. 41:3700, 1981.

30) Reddy B.S. Role of bile metabolites in colon carcinogenesis. Cancer 36:2401, 1975.

31) Reddy B.S. and Watanabe K. Effect of cholesterol metabolites and promoting effects of lithocholic acid in colon carcinogenesis in germ free and conventional F344 rats. Cancer Res. 39:1521, 1979.

32) Goldin B.R. and Gorbach S.L. Effects of antibiotics on incidence

of rat intestinal tumors induced by 1,2-dimethylhydrazine dihydrochloride. JNCI 67:877, 1981.

33) Sloan D.A., Fleiszer D.M., Richards G.K., Murray D. and Brown R.A. Increased incidence of experimental colon cancer associated with long term metronidazole therapy. Am. J. Surg. 145:66, 1983.

34) Kareem M., Fleiszer D.M., Richards G.K., Senterman M. and Brown R.A. Effect of long term metronidazole therapy on experimental colon cancer in rats. J. Surg. Res. 36:547, 1984.

35) Reddy B.S., Furuya K. and Lowenfels A. Effect of neomycin on azoxymethlane-induced colon carcinogenesis in F344 rats. JNCI 73:275, 1984.

36) Lynch H.T., Guirgis H.A., Swartz M.D., Lynch J., Krush M.S. and Kaplan A.R. Genetics and colon cancer. Arch. Surg. 106:669, 1973.

37) Bussey H.J.R. Genetic factors. Recent Results in Cancer Res. 83:45, 1982.

38) Hill M.J. Genetic and Environmental Factors in Human Colorectal Cancer. In: Colon Carcinogenesis, Malt R.A., Williamson R.C.N. (eds). Falk Symposium 31, MTP Press, p. 73-79, 1982.

39) Bussey H.J.R. Familial Polyposis Coli. Johns Hopkins University Press, Baltimore, 1975.

40) Watne A.L., Lai H.Y., Carrier J. and Coppula W. The diagnosis and surgical treatment of patients with Gardner's Syndrome. Surgery 82:327, 1977.

41) Cochet B., Carrel J., Desbaillets L. and Widgren S. Peutz-Jeghers Syndrome associated with gastrointestinal carcinoma. GUT 20:169, 1979.

42) Bussey H.J.R. Polyposis Syndromes. In: Recent Advances in Gastrointestinal Pathology, Wright R. (ed). Saunders, London, p. 345-352, 1980.

43) Lynch H.T., Rozen P. and Schuelke G.S. Heredity and colon cancer polyposis and nonpolyposis variants. CA 35:95, 1985.

44) Lynch H.T. and Lynch P.M. The cancer family syndrome: A pragmatic basis for syndrome identification. Dis. Colon Rectum 22:106, 1980.

45) Moon R.C. and Fricks C.M. Influence of gonadal hormones and age on 1,2-dimethylhydrazine-induced colon carcinogenesis. Cancer 40:2502, 1977.

46) Asano, T. and Pollard M. Strain susceptibility and resistance to 1,2-dimethylhydrazine-induced enteric tumors in germ free rats. Proc. Soc. Exp. Bio. Med. 158:89, 1978.

47) Evans J.T., Haushka T.S. and Mittleman A. Differential susceptibility of four mouse strains to induction of multiple large bowel neoplasms by 1,2-dimethylhydrazine. JNCI 52:999, 1974.

48) Evans J.T., Shows T.B., Sproul E.E., Paolini N.S., Mittleman A. and Haushka T. Genetics of colon carcinogenesis in mice treated with 1,2-dimethylhydrazine. Cancer Res. 37:134, 1977.

49) Diwan B.A., Meier H. and Blackman K.E. Genetic differences in the induction of colorectal tumors by 1,2-dimethylhydrazine in inbred mice. JNCI 59:455, 1977.

50) Diwan B.A., Dempster A.M. and Blackman K.E. Effects of methylazoxymethanol acetate on inbred mice: Influence of genetic factors on tumor induction. Proc. Soc. Exp. Biol. Med. 161:347, 1979.

51) Diwan B.A. and Blackman K.E. Carcinogenic effect of combined treatment of inbred mice with 3-methylcholanthrene and 1,2-dimethylhydrazine. JNCI 64:1491, 1980.

52) Diwan B.A. and Blackman K.E. Differential susceptiblity of 3 sublines of C57BL/6 mice to the induction of colorectal tumors by 1,2-dimethylhydrazine. Cancer Lett. 9:111, 1980.

53) Turusov V.S., Lanko N.S., Krutovskikn V.A. and Parfenov Y.D. Strain differences in susceptibility of female mice to 1,2-dimethylhydrazine. Carcinogenesis 3:603, 1982.

54) Hughes W.L., Bond V.P., Brecher G., Cronkite E.P., Painter R.B., Quastler H. and Sherman F.G. Cellular proliferation in the mouse revealed by autoradiography with tritiated thymidine. Proc. Natl. Acad. Sci. USA 44:476, 1958.

55) Walker B.E. and Leblond C.P. Sites of nucleic acid synthesis in the mouse visualized by radioautography after administration of C^{14}-labelled adenine and thymidine. Exp. Cell Res. 14:510, 1958.

56) Lipkin M. and Quastler H. Cell population kinetics in the colon of the mouse. J. Clin. Invest. 41:141, 1962.

57) Lipkin M., Bell B. and Sherlock P. Cell proliferation kinetics in the gastrointestinal tract of man. I. Cell renewal in colon and rectum. J. Clin. Invest. 42:767, 1963.

58) de Rodriquez M.S.B., Sunter J.P., Watson A.J., Wright N.A. and Appleton D.R. Cell population kinetics in the mucosal crypts of the descending colon of the mouse. Virchows Arch. B (Cell Path.) 29:351, 1979.

59) Chang W.W.L. Renewal of the epithelium in the descending colon of the mouse. III. Diurnal variation in the proliferative

activity of epithelial cells. Am. J. Anat. 131:111, 1971.

60) Hamilton E. Diurnal variation in proliferative compartments and their relation to cryptogenic cells in the mouse colon. Cell Tissue Kinet. 12:91, 1979.

61) Hoff M.B., Chang W.W.L. and Mak K.M. Effect of estrogen on cell proliferation in colonic mucosa of the mouse. Virchows Arch. B (Cell Pathol.) 35:263, 1981.

62) Hoff M.B. and Chang W.W.L. The effect of estrogen on epithelial cell proliferation and differentiation in the crypt of the descending colon of the mouse. A radioautographic study. Am. J. Anat. 158:507, 1979.

63) Fleiszer D.M., Murray D., Richards G.K. and Brown R.A. Effects of diet on chemically induced bowel cancer. Can. J. Surg. 23:67, 1980.

64) Jacobs L.R. Stimulation of Colonic Epithelial Cell Proliferation with Dietary Wheat Bran and its Promoter Effects on 1,2-Dimethylhydrazine Induced Rat Colon Carcinogenesis. The 1983 Workshop on Large Bowel Cancer. Sponsored by the National Large Bowel Cancer Project. June 1983, p. 157-158.

65) Reddy B.S., Narisawa T., Weisburger J.H. and Wynder E.L. Promoting effect of sodium deoxycholate on colon adenocarcinomas in germ free rats. JNCI 56:441, 1976.

66) Lehnert S. Changes in growth kinetics of jejunal epithelium in mice maintained on an elemental diet. Cell Tissue Kinet. 12:239, 1979.

67) Chang W.W.L and Leblond C.P. Renewal of the epithelium in the descending colon of the mouse. I. Presence of three cell populations: Vacuolated-columnar, mucous and argentaffin. Am.

J. Anat. 131:73, 1971.

68) Richards T.C. Early changes in the dynamics of crypt cell populations in mouse colon following administration of 1,2-dimethylhydrazine. Cancer Res. 37:1680, 1977.

69) Tutton P.J.M. and Barkla D.H. Regulation of Cell Kinetics and Colon Cancer. In: Autrup H. and Williams G.M. (eds.). Experimental Colon Carcinogenesis, CRC Press, Boca Raton, p. 199-213, 1983.

70) Lipkin M. Growth Kinetics of Normal Premalignant Gastrointestinal Epithelium. In: Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells. 29th Ann. Symp. on Fundamental Cancer Research. Drwinko B., Humphreys R.M. (eds.). Williams and Wilkins, Baltimore, p. 562, 1977.

71) James J.T., Shamsuddin A.M. and Trump B.F. A comparative study of the normal histochemical and proliferative properties of the large intestine in ICR/Ha and C57BL/Ha mice. Virchows Arch. B (Cell Pathol.) 41:133, 1982.

72) Sunter J.P., Appleton D.R., de Rodriquez M.S.B., Wright N.A. and Watson A.J. A comparison of cell proliferation at different sites within the large bowel of the mouse. J. Anat. 129:833, 1979.

73) Chan P.C., Cohen L.A., Narisawa T. and Weisburger J.H. Early effects of a single intrarectal dose of 1,2-dimethylhydrazine in mice. Cancer Res. 36:13, 1976.

74) Sunter J.P., Appleton D.R. and Watson A.J. Acute changes occurring in intestinal mucosae of rats given a single injection of 1,2-dimethylhydrazine. Virchows Arch. B (Cell Pathol.) 36:47,

1981.

75) Wargovich M.J., Medline A. and Bruce W.R. Early histopathologic events to evolution of colon cancer in C57BL/6J and CF1 mice treated with 1,2-dimethylhydrazine. JNCI 71:125, 1983.

76) Chang W.W.L., Mak K.M. and MacDonald P.D.M. Cell population kinetics of 1,2-dimethylhydrazine induced colonic neoplasm and their adjacent colonic epithelium in the mouse. Virchows Arch. B (Cell Pathol.) 30:349, 1979.

77) Richards T.C. Changes in crypt cell populations of mouse colon during recovery from treatment with 1,2-dimethylhydrazine. JNCI 66:907, 1981.

78) Tutton P.J.M. and Barkla D.H. Cell proliferation in the descending colon of dimethylhydrazine treated rats and in dimethylhydrazine induced adenocarcinoma. Virchows Arch. B (Cell Pathol.) 21:147, 1976.

79) Hattori T., Helpap B. and Gedigk P. Development and cell kinetics of colonic tumors induced in mice by dimethylhydrazine. J. Cancer Res. Clin. Oncol. 105:148, 1983.

80) Lipkin M. and Deschner E. Early proliferative changes in intestinal cells. Cancer Res. 36:2665, 1976.

81) James J.T., Shamsuddin A.M. and Trump B.S. Comparative study of the morphologic histochemical and proliferative changes induced in the large intestine of ICR/Ha and C57BL/Ha mice by 1,2-dimethylhydrazine. JNCI 71:955, 1983.

82) Deschner E.E. Cell Proliferation as a Biological Marker in Human Colorectal Neoplasia. In: Colorectal Cancer: Prevention Epidemiology, and Screening, Winawer S., Schottenfeld D. and Sherlock P. (eds.). Raven Press, New York, p. 133, 1980.

83) Deschner E.E. and Lipkin M. Proliferative patterns in colonic mucosa in familial polyposis. Cancer 35:413, 1975.

84) Deschner E.E. and Maskens A.P. Significance of the labelling index and labelling distribution as kinetic parameters in colorectal mucosa of cancer patients and DMH treated animals. Cancer 50:1136, 1982.

85) Maskens A.P. and Deschner E.E. Tritiated thymidine incorporation into epithelial cells of normal-appearing colorectal mucosa of cancer patients. JNCI 58:1221, 1977.

86) Ames B.N., Durston W.E., Yamasaki E. and Lee F.P. Carcinogens are mutagens: A simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. USA 70:2281, 1973.

87) Heddle J.A., Blakey D.H., Duncan A.M.V., Goldberg M.T., Newmark H., Wargovich M.J. and Bruce W.R. Micronuclei and related nuclear anomalies as a short term assay for colon carcinogens. Banbury Rep. 13:367, 1982.

88) Goldberg M.T., Blakey D.H. and Bruce W.R. Comparison of the effects of 1,2-dimethylhydrazine and cyclophosphamide as micronucleus incidence in bone marrow and colon. Mutat. Res. 109:91, 1983.

89) Blakey D.H., Duncan A.M.V., Wargovich M.J., Goldberg M.T., Bruce W.R. and Heddle J.A. Detection of nuclear anomalies in the colonic epithelium of the mouse. Cancer Res. 45:242, 1985.

90) Wargovich M.J., Goldberg M.T., Newmark H.L. and Bruce W.R. Nuclear aberrations as a short term test for genotoxicity to the colon: Evaluation of nineteen agents in mice. JNCI 71:133,

1983.

91) Maskens A.P. Significance of the karyorrhectic index in 1,2-dimethylhydrazine carcinogenesis. Cancer Lett. 8:77, 1979.

92) Wyllie, A.H., Kerr, J.F.R. and Currie A.R. Cell death: The significance of apoptosis. Int. Rev. Cytol. 68:251, 1980.

93) Heddle J.A. and Carrano A.V. The DNA content of micronuclei induced in mouse bone marrow by gamma irradiation: Evidence that micronuclei arise from acentric chromosomal fragments. Mutat. Res. 44:67, 1977.

94) Schmid W. The micronucleus test. Mutat. Res. 31:9, 1975.

95) Duncan A.M.V., Heddle J.A. and Blakey D.H. Mechanisms of induction of nuclear anomalies by gamma-radiation in the colonic epithelium of the mouse. Cancer Res. 45:250, 1985.

96) Deschner E.E., Long F.C., Hakissian M. and Herrman S.L. Differential susceptibility of AKR, C57BL/6J and CF1 mice to 1,2-dimethylhydrazine-induced colonic tumor formation predicted by proliferative characteristics of colonic epithelial cells. JNCI 70:279, 1983.

97) Deschner E.E., Long, F.C., Hakissian M. and Cupo S.H. Differential susceptibility of inbred mouse strains forecast by acute colonic proliferative response to methoxymethanol. JNCI 72:195, 1984.

98) Genetic Control of Natural Resistance to Infection and Malignancy. Skamene E., 'Konghavn P.A.L. and Landry M. (eds). Academic Press, New York, 1980.

99) Stevenson M.M., Kongshavn P.A.L. and Skamene E. Genetic linkage of resistance to listeria monocytogenes with macrophage inflammatory responses. J. Immunol. 127:402, 1981.

100) Leblond C.P., Messier B. and Kapriwa B. Thymidine H^3 as a tool for the investigation of the renewal of cell populations. Lab. Invest. 8:296, 1959.

101) Deschner E.E. Early proliferative defects induced by six weekly injections of 1,2-dimethylhydrazine in epithelial cells of mouse distal colon. Z. Krebsforsch. 91:205, 1978.

102) Grab D.J. and Zedeck M.S. Organ specific effects of the carcinogen methylazoxymethanol related to metabolism by nicotinauride adenine dinucleotide-dependent dehydrogen dehydrogenases. Cancer Res. 37:4182, 1977.

103) Cooper H.K., Buecheler J. and Kleihues P. DNA alkylation in mice with different susceptibility to 1,2-dimethylhydrazine induced colon carcinogenesis. Cancer Res. 38:3063, 1978.

104) Kanagalingam K. and Balis M.E. In vivo repair of rat DNA damage by alkylating agents. Cancer 36:2364, 1975.

105) Chang W.E.L. and Nadler N.J. Renewal of the epithelium in the descending colon of the mouse. VI. Cell population kinetics of vacuolated-columnar and mucous cells. Am. J. Anat. 144:39, 1975.

106) Hawks A. and Magee P.N. The alkylation of nucleic acids of rat and mouse in vivo by carcinogen 1,2-dimethylhydrazine. Br. J. Cancer 30:440, 1974.

107) Rogers K.J. and Pegg A.E. Formation of O^6-methylguanine by alkylation of rat liver, colon and kidney DNA following administration of 1,2-dimethylhydrazine. Cancer Res. 37:4082, 1977.

108) James J.T. and Autrup H. Methylated DNA aducts in the large intestine of ICR/Ha and C57BL/Ha mice given 1,2-

dimethylhydrazine. JNCI 70:541, 1983.

109) Mak K.M. and Chang W.W.L. Inhibition of DNA synthesis by 1,2-dimethylhydrazine and methylazoxymethanol acetate in rabbit colon mucosa in organ culture. JNCI 61:799, 1979.

110) Zedeck M.D., Grab D.J. and Sternberg S.S. Difference in the acute response to various segments of rat intestine to treatment with the intestinal carcinogen methylazoxymethanol acetate. Cancer Res. 37:32, 1977.

111) Maskens A.P. and Dujardin-Loits R.M. Correlation between karyorrhectic index in colon crypts and tumor yield in dimethylhydrazine treated rats. Proc. Am. Assoc. Cancer Res. 19:151, 1978.

112) Ronen A. and Heddle J.A. Site specific induction of nuclear anomalies (apoptotic bodies and micronuclei) by carcinogens in the mouse. Cancer Res. 44: 1536, 1984.

113) Chang W.W.L. Degenerative behaviour of epithelial cells in the colonic crypt of the mouse following administration of colonic carcinogen 1,2-dimethylhydrazine. Cancer Lett. 13:111, 1981.

114) Duncan A.M.V., Ronen A. and Blakey D.A. Diurnal variation in the response of gamma-ray induced apoptosis in the mouse intestinal epithelium. Cancer Lett. 21:163, 1983.

115) Glickman L.T. and Fleiszer D.M. The proliferative characteristics of colonic crypt cells as predictors of subsequent tumor formation. Surg. Forum, Vol. 36, 1985 (In Press).