DIETARY EFFECTS ON SPONTANEOUS GENETIC DAMAGE AND SOMATIC MUTATION FREQUENCIES

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A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

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ABSTRACT

Epidemiological and animal studies have established a correlation between diet and the incidence of many cancers, yet no specific foods or dietary factors have been identified to account for this correlation. Somatic mutations have a critical role in carcinogenesis. The measurement of spontaneous genetic damage using *in vivo* mutation assays provides an alternative approach for studying dietary correlations. Most spontaneous mutations arise during early development, when high rates of cell proliferation occur. Greater sensitivity of the dividing cell to endogenous and exogenous factors suggests that this is the time when dietary anti-mutagens and anti-carcinogens are most needed and would be most effective. Therefore, altering the diet of the mother and infant in early life could reduce the frequency of somatic mutations and the resulting cancers later on.

Folic acid deficiency is associated with an increase in chromosomal aberrations in adult rodents and humans. The frequency of mutations at the *lacZ* transgene were no different between mice whose mothers were fed low folate and those fed high folate diets during pregnancy. Similar results were observed with respect to the incidence of micronuclei in red blood cells. The minimal requirement for folic acid appears to be sufficient for limiting genetic damage so that increased consumption is of no greater benefit.

Using flow cytometry to measure micronuclei in peripheral blood erythrocytes of mice, 26 vegetables, fruits, and grains were screened for their effect on spontaneous

chromosome damage. A significant reduction in the spontaneous frequency of micronuclei was observed in mice maintained on flaxseed supplemented diets for 44 days, suggesting an anti-mutagenic property for flaxseed.

Dietary supplementation during development with one of 10 foods, did not lower the frequency of mutations at the *lacZ* transgene below those observed in mice fed AIN-93G control diet. The mutation frequencies observed were significantly lower however, than those of laboratory rodent chow. We postulate that nutritional deficiencies in the standard diet account for the increase in mutation frequencies.

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LIST OF ABBREVIATIONS

bp	base pair
DNA	deoxyribonucleic acid
DBA	*Dolicho biflorus* agglutinin
Dlb-1	*Dolichos biflorus* lectin binding
EDTA	disodium ethylene diamine tetraacetate
KCl	potassium chloride
LB	Lauria Bertani medium
$MgSO_4$	magnesium sulphate
MN	micronucleus
NaCl	sodium chloride
NCE	normochromatic erythrocyte
p-gal	phenyl-β-*D*-galactopyranoside
PBS	phosphate buffered saline
RBC	red blood cell
RET	reticulocyte
RNA	ribonucleic acid
SEM	standard error of the mean
SDS	sodium dodecyl sulfate
TE	tris-EDTA buffer
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Carcinogenesis is a highly complex process with many possible causes, including inherited and somatic mutations, environmental and dietary factors (Ames and Gold, 1991; Ames et al., 1995; Knudsen, 1996). The idea that mutations are a necessary requirement is not novel; Boveri (1929) introduced the somatic mutation theory of carcinogenesis close to the beginning of this century. The theory states that the conversion of normal cells to malignant cancer cells is the result of genetic damage. It is now known that this transformation proceeds by sequential steps, with the accumulation of mutations in several critical genes (Vogelstein et al., 1989; Vogelstein and Kinzler, 1993). Inherited mutations are insufficient for cancer initiation; it is the somatic mutations acquired during the lifetime of the individual that play a more important role.

A proportion of these somatic mutations is unavoidable, arising from DNA damage caused by normal functioning of the cell (Ames and Gold, 1990). Endogenous oxidants induce genetic damage, which is converted to stable mutations during cell division. For this reason, a dividing cell is much more at risk of mutating than is a quiescent cell. Since rapid cell division and growth characterize early development the rate of mutant accumulation during this period may be significant (Zhang et al., 1995; Heddle et al., 1996; Paashuis-Lew and Heddle, 1997).

A second important area of cancer research is the study of diet and its relationship to carcinogenesis (Ames et al., 1995). While diet is considered to be a major risk factor in the etiology of many human cancers, several dietary constituents have been recognized as

protective against these same cancers (Fiala et al., 1995; Wargovich, 1997). Previous experimental studies of nutrition and cancer have focused on adult rodents and humans ignoring the idea that the young may be more susceptible to carcinogenesis because of the high mutation rate during early life. In doing so, they have also failed to consider that dietary anti-mutagens and anti-carcinogens may be most effective during this developmental period. The possibility that the correlation between diet and cancer risk is the result of nutritional factors modifying somatic mutation rates during development therefore warrants study as a means of reducing the incidence of many cancers. Examination of this hypothesis requires an understanding of how spontaneous mutations accumulate, their role in cancer, and preventative mechanisms of dietary anti-mutagens and anti-carcinogens.

1.2 SPONTANEOUS MUTATIONS IN DEVELOPMENT AND CANCER

Spontaneous mutations are considered to be those which occur in the absence of exogenous mutagenic factors (Loeb, 1991). This definition covers mutations that arise in a variety of different ways including errors of DNA replication, repair and recombination, deamination and depurination of DNA bases, and exposure to endogenous mutagens. DNA damage caused by oxygen radicals is thought to be the most significant endogenous damage. The number of oxidative hits to DNA per cell per day is estimated, based on urinary levels of DNA adducts, to be 10^5 in the rat and approximately 10^4 in humans (Cathcart et al., 1984; Ames and Gold, 1990; Fraga et al., 1991).

The frequency at which spontaneous mutations occur is in part related to the age of the individual. Mutant frequencies at the hypoxanthine phosphoribosyl-transferase (*hprt*) gene were measured in T-lymphocytes from healthy human individuals in different age groups (Finette et al., 1994; Robinson et al., 1994). Mutations within the *hprt* gene, which confer resistance to 6-thioguanine, were found to accumulate more rapidly between birth and adulthood than later periods in life. Two additional studies examining spontaneous mutant frequency in transgenic mice also indicated a steep accumulation of mutants during development (Lee et al., 1994; Ono et al., 1995). More recently, mutant accumulation was measured in 12.5 and 15.5 days post conception (dpc) mouse fetuses and a steep increase in mutant frequencies was observed during this developmental period (Paashuis-Lew and Heddle, 1997). Results of this work in conjunction with those of previous studies, indicate that approximately 1/3 of mutants accumulate before 12.5 dpc, 1/3 from 12.5 to 1 month and the remaining 1/3 during adulthood (Figure 1; Paashuis-Lew and Heddle, 1997).

The correlation between age and rate of mutant accumulation is possibly a consequence of the number of cell divisions occurring at that age. Fetal development is characterized by high rate of cell proliferation, more so than at any other time in life. This is evident by the rapid increase in weight during this period (Crispen, 1975). Regular cell division is limited only to a small number of tissues during adulthood, such as the skin, intestinal lining and hemapoietic system. The spontaneous mutation rate per cell division at a particular locus is constant throughout life producing a linear increase in

mutant frequency when plotted as a function of the number of cell divisions (Figure 2; Schaaper and Dunn, 1991; Heddle et al., 1996; Kowald and Kirkwood, 1996). Since most cell divisions that "make" a mouse occur before birth, if mutant accumulation is plotted as a function of weight of the animal or cell number, both which can be used as a substitute for age, a non-linear relationship is observed (Figure 3; Heddle et al., 1996). The mutant frequency at conception can be plotted as 0, since an inherited mutation would produce a high mutant frequency known as a "jackpot". The slope of the curve between conception and birth is far steeper than at any other region on the graph.

A high rate of cell division may increase mutagenesis for several reasons. First, it may increase the likelihood of DNA replication errors. Second, the time interval available for repair is shorter, increasing the probability that some DNA damage is converted to mutations at replication. Finally, open strands of replicating DNA may expose nucleotides to genotoxic agents that are present within the cell and therefore, increases the potential of attack (Ames and Gold, 1991).

The transformation of a normal cell to an initiated state requires an irreversible genetic alteration of the cellular DNA at a critical site (van Poppel and van den Berg, 1997). This may occur spontaneously by a mechanism described earlier, or be induced by a carcinogenic agent. Selective clonal expansion of the cell converts it from an initiated to pre-malignant cell ("promotion"; van Poppel and van den Berg, 1997). This may also occur spontaneously or by tumor promoters but involves no additional genetic damage and is considered reversible. According to most theories, promoters are

generally not carcinogenic on their own but may affect gene expression resulting in the impaired differentiation of the initiated cell. Finally, progression to a malignant tumor is a consequence of further damage to DNA and a breakdown in normal control mechanisms (van Poppel and van den Berg, 1997). Dietary nutrients may exert their protective effects at any stage of carcinogenesis in any number of ways.

1.3 DIETARY ANTI-CARCINOGENIC AGENTS

Approximately 35% of all cancers are attributable to dietary factors (Doll and Peto, 1981). The identification of dietary constituents that act as initiators and promoters of the carcinogenic process has also led to the identification of naturally occurring agents that inhibit it. Many are biologically active compounds of plant origin, "phytochemicals", that have diverse plant functions as antioxidants, pigments or defense against disease (Wargovich, 1997). Included in this definition are vitamins and trace elements of which humans rely on exogenous sources for supplementation. The inverse association between the consumption of fruits and vegetables and cancer risk has been shown consistently by numerous epidemiological and experimental studies (Steinmetz and Potter, 1991; Block et al., 1992).

The influence of dietary anti-carcinogens on the carcinogenic process can be categorized into four general mechanisms. They may: (1) modify carcinogen activation by inhibiting Phase I enzymes; (2) modify carcinogen detoxification via Phase II

enzymes; (3) scavenge DNA reactive agents; and (4) suppress abnormal proliferation (Wargovich, 1997).

1.3.1 Dietary Factors that Modify Carcinogen Activation

Many foreign chemicals that enter the body are lipid-soluble, a property that enables them to penetrate biological membranes. The cytochrome P-450-dependent mono-oxygenases (Phase I enzymes) catalyze a variety of reactions (oxidation, reduction, hydrolysis) in which a polar group is added to the molecule thereby increasing solubility and aiding in excretion of the chemical from the body (Fiala et al., 1985). In some cases however, the metabolites ("ultimate carcinogen") of biotransformation reactions are more reactive than their parent compounds ("pro-carcinogen"). Many naturally occurring anti-carcinogens act on the enzymes involved in activation by down-regulating enzyme levels and/or directly inhibiting their catalytic activities (Fiala et al., 1985; Zhang and Talalay, 1994).

Cruciferous vegetables such as broccoli, cabbage and brussel sprouts contain two groups of potent inhibitors of Phase I enzymes, the isothiocyanate and indole compounds. Isothiocyanates are among the most effective chemopreventive agents known, exhibiting protection against cancer in the lung, mammary gland, esophagus, liver, small intestine, colon and bladder (Fiala et al., 1985). Benzyl isothiocyanate (BITC) for example, can inhibit 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary tumors and

mouse lung tumors induced by the environmental lung carcinogen benzo[a]pyrene (B*a*P) (Morse, 1989; Hecht, 1995).

Indole-3-carbinol and related indoles, 3,3'-diindolylmethane and indole-3-acetonitrile, are formed in cruciferous vegetables from the hydrolysis of glucosinolate. In experimental animals, they are protective against a variety of tumors induced by carcinogens including B*a*P and polycyclic aromatic hydrocarbons, suppressing carcinogen activation by P-450 enzymes (Wattenberg and Loub, 1978).

In allium vegetables, such as onions, garlic and chives, a number of pharmacologically active, organosulfur compounds are formed when precursors are acted upon by the enzyme allinase. These alkyl sulfides and disulfides, which give the vegetables their flavour, can inhibit Phase I enzymes both *in vitro* and *in vivo*. When given to mice, diallyl sulfide was found to inhibit the incidence of colorectal tumors induced by 1,2-dimethylhydrazine (DMH), a known colon carcinogen in rodents (Wargovich, 1987).

Although it does not act directly on one of the Phase I enzymes, vitamin C (ascorbic acid) has a role in preventing the first step of carcinogenesis. Vitamin C has long been recognized as an anticarcinogenic agent with widespread biological function (Block, 1991). Found in high quantities in fruits and vegetables, one role of this vitamin is in preventing the formation of mutagenic nitrosamines from nitrite precursors. The majority of nitrosamines are potent organ-specific carcinogens in experimental animals (Mirvish, 1986). Nitrites are constituents of a number of dietary foods particularly,

salted, pickled or smoked foods. In the gastrointestinal (GI) tract, these chemicals react with secondary amines to form nitrosamines. Vitamin C can react with nitrite and convert it to nitrous oxide thereby preventing the formation of mutagenic nitrosamines and reducing cancer occurrence, particularly in the stomach (Mirvish, 1986).

Vitamin E has also been found to prevent nitrosamine formation in gastrointestinal tract by inhibiting *N*-nitrosation reactions (Mirvish, 1986). Dietary sources of this vitamin include whole grains, wheat germ, seeds, nuts and green vegetables. The most active form in foods is α-tocopherol. The combined use of vitamin C and vitamin E has been shown to have a synergistic effect in the prevention of nitrosamine synthesis (Lathia and Blum, 1989).

1.3.2 Dietary Factors that Modify Carcinogen Detoxification

The Phase II enzymes glutathione transferase and peroxidase, together with cellular levels of glutathione (GSH), provide a second line of defense against carcinogenic agents. GSH reacts with electrophiles such as pro-carcinogens and ultimate carcinogens to yield GSH conjugates that are inert and more easily excreted (Fiala et al., 1985). Increased rates of detoxification of chemical carcinogens by GSH transferases can be stimulated by dietary components and therefore, decrease the probability of carcinogen interaction with DNA and other critical nucleophiles.

The isothiocyanate and sulfide compounds discussed earlier have dual roles as active inhibitors of Phase I enzymes and inducers of Phase II enzymes. Some, such as

sulforaphane, a potent isothiocyanate recently isolated from broccoli, are monofunctional, acting only on one of the two groups of enzymes. Others mediate both Phase I and Phase II enzymes in a cooperative fashion (Zhang et al., 1992).

Studies have demonstrated anti-carcinogenic effects of polyphenols, dietary constituents of various fruits, vegetables, and nuts and more recently, green tea. A 2% solution of green tea, given as the sole source of drinking water, resulted in a significant reduction in both lung and stomach tumors induced by B*a*P (Wang et al., 1992). Green tea and its components are also protective against tumors in the small intestine, colon, liver, pancreas and mammary gland. The mechanism of action involves, as with other agents in this category, the enhancement of Phase II enzyme activity (Stoner and Mukhtar, 1995).

1.3.3 Dietary Factors that Scavenge DNA Reactive Molecules

The normal metabolism of the cell can inadvertently lead to the production of oxidants or free radicals that have the potential of causing extensive damage to cellular and genetic structures. Free radicals are highly reactive species because of one or more unpaired electrons (Thompson, 1994). They can initiate lipid peroxidation by reaction with polyunsaturated fatty acids, inactivate proteins and enzymes by reactions with amino acids, and damage RNA and DNA by reaction with guanine (Thompson, 1994). Enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase, exist to protect the cell from oxidative damage. Dietary antioxidants are

exogenous sources of defense and have been implied in both initiation and promotion phases in animal studies. They may prevent the formation of free radicals and singlet oxygen molecules or scavenge and combine with these molecules and stablize their reactivity (Wargovich, 1997).

The tumor-inhibiting effect of the polyphenolic compounds discussed earlier may also be mediated through an alternative mechanism. Although they act on Phase II enzymes, they are more potent as antioxidants, inhibiting chemically-induced lipid peroxidation and scavenging reactive metabolites (Stoner and Mukhtar, 1995). This property coupled with their effect on carcinogen detoxification, make them effective against both initiation and promotion stages of carcinogenesis.

Carotenoids, the most recognized antioxidants and of which β-carotene (provitamin A) is the most known, are present in dark green leafy vegetables and in yellow and orange vegetables and fruits. Numerous studies have demonstrated their capacity to deactivate mono-oxygen molecules and trap free radicals (Buring and Hennekens, 1995; Gerster, 1995). Both vitamin C and E are included with the carotenoids as intracellular antioxidants. Vitamin E, a fat-soluble vitamin that is an integral component of cellular membranes, can protect membrane lipids from peroxidation. An insufficient level of vitamin C increases spontaneous oxidative damage to sperm DNA (Fraga et al., 1988). Supplementation of vitamin C (100 mg/day), E (280 mg/day) and β-carotene (25 mg/day) in the diets of smokers and non-smokers for

20 weeks, significantly decreased endogenous oxidative damage in lymphocytes, supporting the cancer-protective role of these dietary compounds (Duthie et al., 1996).

Although selenium, a trace component of the diet, is not an antioxidant, it may be a precursor of a compound or a complex capable of carrying out antioxidative functions. It is a known cofactor for glutathione peroxidase, an enzyme that protects against oxidative damage and acts synergistically with vitamin E in the inhibition of lipid peroxidation (Wattenberg, 1985). Given in drinking water (4 ppm), selenium can inhibit the induction of colon tumors in rats by 1,2-dimethylhydrazine (DMH; Jacobs et al., 1981). The mechanism by which selenium exerts its inhibitory effect is not completely understood.

1.3.4 Dietary Factors that Reverse Abnormal Proliferation

A characteristic feature of cancer is the loss of a cell's ability to control proliferation, leading to excessive cell division. Recently, vitamin A (retinol) and its derivatives have been used as cancer preventive agents because they regulate normal cell growth and differentiation in vertebrates. They act through retinoid receptors found in the nucleus, which can bind *cis* regulatory sequences and cause changes in gene expression and consequential changes in cell growth and differentiation (Love and Gudas, 1994). Studies examining the effect of retinol deficiency, have shown that low serum vitamin A can enhance the development of chemically-induced tumors in

experimental animals, providing evidence for a role of vitamin A in carcinogenesis (Graham, 1984).

A similar role in chemoprevention has been suggested for calcium since it is essential for a wide variety of cell functions, most importantly the regulation of the cell cycle (Whitfield et al., 1995). Increasing levels of calcium *in vitro* decreases proliferation and induced cell differentiation in epithelial cells, mammary cells and colon cells. Similar effects were observed *in vivo* in both rodent and human colon. Supplemental calcium in animal diets effectively decreased the hyperproliferation of colon epithelial cells associated with ingestion of high fat diets (Lipkin and Newmark, 1995). Binding of calcium to soluble fatty acids and bile acids in colonic lumen decreases mucosal cell toxicity by these compounds.

Flavonoids, common plant pigments found in most fruits and vegetables, display a variety of biological functions, including antioxidant activity. They are however, more recognized for their ability to inhibit tumor growth, especially in the gastrointestinal (GI) tract, by suppressing cell proliferation induced by chemical carcinogens (Tanaka et al., 1997). It has been suggested that the antiproliferative effects of flavonoids may be due to suppression of ornithine decarboxylase (ODC). The rate of cell proliferation in several tissues has been correlated with the activity of ODC, an enzyme involved in polyamine biosynthesis. Agents which inhibit ODC activity are effective tumor inhibitors (Pegg, 1988).

Although protease inhibitors and dietary fiber do not mediate their anti-carcinogenic effects through one of the mechanisms listed above, their role in cancer prevention deserves attention. Green tea and grains including barley, wheat, oats and rye contain protease inhibitors, with seeds and legumes as particularly rich sources. The actions of proteases produced by cancer cells, leads to destruction of the extracellular matrix, cellular detachment and subsequent local invasion (Richardson, 1977). Dietary inhibitors act by competitively inhibiting proteases via the formation of complexes and blocking protein absorption thereby suppressing tumor progression (Fiala et al., 1985). Anti-protease activity can protect against both spontaneous and chemically-induced tumors in animals and may function to inhibit both initiation and promotion stages of carcinogenesis (Kennedy, 1994).

Dietary fiber found in vegetables, fruits, legumes, nuts, seeds and unrefined grains, comprises a heterogenous group of carbohydrate compounds including cellulose, hemicellulose, pectin and a noncarbohydrate substance, lignin, that are resistant to digestion in the GI tract (Fiala et al., 1985). The addition of fiber to the diet can increase fecal bulk and decrease intestinal transit rates. Absorption of any potential carcinogens present in the intestine are diluted by the bulking effect and faster transit through the intestine, decreases mucosal contact time (Klurfeld, 1992). The protective effect of fiber depends on composition and source of fiber in the diet. Insoluble fibers such as cellulose, tend to decrease transit time and increase fecal bulk; soluble fibers such as pectin, are less effective since they are well fermented by intestinal bacteria.

The direct binding of dietary fiber to bile acids in the colon may provide two additional mechanisms for modulating carcinogenesis. Bile acids themselves have been suggested to indirectly damage DNA by acting as co-mutagens to chemicals such as DMH (Wilpart, 1983). The binding of fiber to bile acids would thus prevent any such effect. In addition, decreased levels of free bile acids, would decrease enterohepatic circulation of carcinogens and consequently, increase their excretion from the body (Klurfeld, 1992).

1.4 FOLIC ACID

There is accumulating evidence from animal and cell models of a possible role for folic acid as a cancer-preventing agent. A component of the vitamin B complex, folate is associated with lower cancer risk in epidemiological studies, and a deficiency of folic acid in experimental studies, results in DNA damage that resembles the damage found in cancer cells (Krumdieck, 1983). Humans are dependent on dietary sources of folate with the richest sources being legumes such as soybeans, peas and lentils, cruciferous vegetables and whole grains. Folic acid deficiency is believed to be the most common vitamin deficiency in the world, particularly among pregnant women due to the increased folate demands for the growing fetal and maternal tissues (Steegers-Theunissen, 1995). Deficiency of the vitamin in adults is linked to an increase in chromosomal aberrations (Everson et al., 1988; MacGregor, 1990) and during pregnancy, it is associated with an elevated incidence of birth defects (Nelson et al., 1955; Milunsky et al., 1989).

Mammalian cells have an absolute requirement for folic acid. The prime function of folate is to provide one-carbon fragments for the biosynthesis of purines, in particular thymine, as well as for the synthesis of other compounds such as methionine (Steegers-Theunissen, 1995). A deficiency of folic acid leads to alterations in the pool of nucleotides available for DNA and RNA synthesis. Blockage of thymine synthesis results in thymine depletion and expansion of the cytosine and uracil pools, with the increase in the latter pool causing uracil to become incorporated into DNA at sites designated for thymine (Jennings, 1995). Since uracil is not a normal component of the DNA structure, any uracil that is incorporated is quickly removed by the enzyme uracil-DNA glycosylase, leaving an apyrimidinic site, which is then acted upon by an apurinic/apyrimidinic endonuclease, followed by excision and repair (Goulian et al., 1980). Attempts to fill in gaps left during repair may result in incorporation of additional uracil, thereby re-initiating the process of removal by DNA glycosylase and further repair. This cycle of uracil removal, re-incorporation and re-removal may result in extensive degradation of DNA, ultimately leading to double-strand breaks or other disruptions of the DNA structure (Goulian et al., 1980). Folate deficiency in Swiss mice was shown to increase the incidence of chromosome breakage, indicated by increased micronucleus formation in peripheral blood erythrocytes (MacGregor et al., 1990).

The absence of the thymine methyl group in the DNA strand interferes with the normal interactions between DNA and the proteins that maintain the condensed structure of the chromosomes. Decondensed chromosomes may be more susceptible to further

DNA damage than properly condensed chromosomes (Jennings, 1995) and may explain why dietary deficiency of folic acid enhances carcinogen-induced carcinogenesis (Rogers and Newberne, 1975; Branda and Blickensferfer, 1993).

A secondary function of folic acid is in gene regulation. Methylation of cytosine residues at CpG dinucleotides within DNA, plays an important role in the regulation of mammalian gene expression. Usually, cytosine methylation is associated with gene inactivation whereas a decrease in methylation results in gene activity (Jones and Buckley, 1990). The methyl group on the 5-carbon position of cytosine residues is derived from *S*-adenosylmethionine (SAM). Folic acid is a cofactor in the metabolic cycle that maintains the supply of SAM (Jennings, 1995). Folate deficiency may disrupt SAM synthesis and contribute to the improper expression of some genes and the disruption of normal growth-control mechanisms (Jennings, 1995). Hypomethylation of DNA, at specific genes and oncogenes, has been detected in tumor tissue, including malignant colon neoplasms. This is in comparison with normal tissue where, normal methylation patterns were observed (Feinberg and Vogelstein, 1983; Goelz et al., 1985).

1.5 EVALUATION OF DIET AND CANCER DATA

The enormous volume of data that has accumulated over the last few decades examining the relationship between diet and cancer have come from two main sources, human and animal studies. The evaluation and acceptance of correlations identified from

these studies requires that one understand the strengths and limitations in the methods used to obtain the data.

Epidemiological studies have been and continue to be a primary source for data. Associations based on epidemiological evidence generally are of greater importance than those derived by other methods because the relationship of diet to cancer in humans is directly addressed (Thompson, 1993). However, the interpretation of epidemiological data may not be so straightforward. As information about diet is usually obtained by means of a questionnaire on the frequency of consumption of various foods, the data is often imprecise and subject to biases especially if the participant or interviewer is aware of the hypothesis. Interpretation is complicated by one additional factor. Most people are unable to accurately recall their dietary intake over a period of time. It is therefore impossible to be confident that a particular factor is the causative or protective agent (Thompson, 1993).

Animal studies provide a direct means to validate a cause and effect relationship that is suggested by epidemiological data. They give the investigator a level of control and an ability to manipulate key dietary variables that is not possible with human studies (Willet and MacMahon, 1984). Although animal experimentation is valuable to diet and cancer research, extrapolation to humans is complicated by the fact that animals used in nutrition studies are usually treated with high doses of a known carcinogen. The human population is normally not exposed to many of the carcinogens used in such studies.

Most carcinogenic agents in the environment are natural and human exposure is at levels far below the doses given to animals.

Very few animal studies have been carried out to examine spontaneous carcinogenesis since such tumors are rare in laboratory rodents. There are many genetic events in a single animal, which makes it possible in principle to measure dietary effects on spontaneous events using various mutation assays.

1.6 MUTATIONAL ASSAYS

Since the pioneering studies of H. J. Muller with Drosophila (1927) at the beginning of this century, numerous assay systems have been developed for the detection of spontaneous and induced mutations, both *in vitro* and *in vivo*. The *Salmonella*/mammalian-microsome assay introduced by Ames, was one of the first tests used to correlate mutagenicity and carcinogenicity (Ames et al., 1973; Ames et al., 1975). Chemical mutagens are detected based on their ability to revert *Salmonella* strains containing base pair substitution or frameshift mutations in the histidine operon to wild-type phenotype. To this day, it is still the most widely used mutation screen. There are several drawbacks to the Ames assay however. It uses an exogenous metabolic activation system (S-9 rat liver extract) to allow for the metabolism of some chemical substances. In addition, the assay is limited in its applicability to detect tissue-specific mutagenicity (Tennant et al., 1994).

In vitro assays using mammalian cells were utilized to account for differences in genome organization and genetic complexity between prokaryotes and eukaryotes. The usefulness of these test systems however, is also limited. Analysis of mutations at target genes such as *hprt* and thymidine kinase (*tk*) is restricted to only one or a few cell types since not all cells can be cultured. It is for this same reason that comparative study of gene mutations in various organs and tissues cannot be carried out (Gossen and Vijg, 1993). Furthermore, many cell lines lose specific properties upon culturing which is likely to have effects on the assay's ability to detect a particular mutation (Lohman et al., 1987). Although they can provide a rapid and relatively inexpensive tool for biologic research, *in vitro* assays do not accurately reflect the human situation and as a result, improvements in testing have focused on turning from *in vitro* assays to *in vivo* studies.

The mouse specific-locus test was the first *in vivo* mammalian mutation detection system to be developed (Russell, 1951). The assay measures the transmission of induced germ-line mutations from parent to offspring. Seven recessive markers are used for detecting new mutants among progeny of mutagen-treated homozygous dominant males and homozygous recessive females. Time, cost, and tissue specificity limit the applicability of the assay however.

Two additional *in vivo* assays, which are used in this thesis, are discussed below.

1.6.1 Micronucleus Assay

The micronucleus assay is a simple and rapid *in vivo* cytogenetic test for detecting chromosome aberrations in a variety of cell types (Heddle, 1973; Schmid, 1975). The assay was first developed for bone marrow and peripheral blood erythrocytes but has been extended for *in vitro* study in human lymphocytes, and skin, liver, lung and colonic epithelial cells (Heddle, 1973; Schmid, 1975; Countryman and Heddle, 1976; Tates et al., 1980; Schlegel and MacGregor, 1982; Fenech and Morley, 1985; Heddle et al., 1990). Bone marrow and peripheral blood are the more widely chosen mediums for study.

Micronuclei arise as a result of chromosomal breakage, or whole chromosome loss due to a dysfunctional spindle apparatus (Heddle, 1973; Schmid, 1975). These lagging fragments and chromosomes are not incorporated into daughter nuclei during cell division and instead, form their own extranuclear body of DNA within the daughter cells (Heddle, 1973; Schmid, 1975). Micronuclei can be generated as a result of damage by some exogenous chemical or physical agent, or they can arise spontaneously as a consequence of endogenous factors. Using DNA specific dyes, one can quantify the number of cells containing micronuclei, for instance in reticulocyte and mature erythrocyte cell populations, and get a measure of genetic damage caused by recent or long-term exposure. As reticulocytes represent the immature cells of the blood, they are indicators of recent damage and can be differentiated from mature erythrocytes based on their high RNA content or high transferrin receptor content (Hayashi et al., 1983; Dertinger et al., 1996).

Since the time the micronucleus assay was introduced, the usefulness and sensitivity of the assay has been extended. Determination of micronucleus size by various methods including the use of fluorescent dyes, has allowed for speculation of the mode by which the micronucleus was generated, either by clastogenic or aneugenic action (Heddle and Carrano, 1977; Grawé et al., 1993). Detection of centromeric regions using kinetochore-specific antibodies or fluorescent *in situ* hybridization with DNA probes specific for centromeric sequences, are also used for the same purpose (Hayashi et al., 1994; Grawé et al., 1994). The advent of automated methods such as high speed flow cytometry, has slowly eliminated manual scoring. The analysis of a larger number of cells in a shorter period of time increases the efficiency, sensitivity and accuracy of the measurements. In addition, cells can be sorted based on size or DNA content of micronuclei (Tometsko et al., 1993; Dertinger et al., 1996; Grawé et al., 1997).

1.6.2 The *Dlb-1* locus

The *Dlb-1* (*Dolichos biflorus* lectin binding) locus has been developed as a marker for studying the incidence and accumulation of spontaneous and induced mutations within the small intestine (Winton et al., 1988). Two alleles, "a" and "b", at the *Dlb-1* locus, determine the tissue-specific pattern of expression of a glycoprotein binding site for the lectin, *Dolichos biflorus* agglutinin (DBA). The dominant Dlb-1^b allele confers positive binding ability for DBA lectin in the intestinal epithelium whereas, the Dlb-1^a allele results in expression of DBA receptors on endothelial cells and no

positive binding on intestinal epithelium. Certain strains of inbred mice such as SWR, are homozygous for Dlb-1^a allele and therefore, lectin binding is specific only to the vascular endothelium. In contrast, other strains such as C57Bl/6, are homozygous for Dlb-1^b allele. Lectin binding is restricted therefore to the intestinal epithelial cells (Ponder and Wilkinson, 1983; Ponder et al., 1985). Specificity of binding between lectin and receptor is based on recognition of N-acetylgalactosamine residues (Sato and Muramatsu, 1985).

The presence of binding sites within the small intestine can be detected by staining with a DBA-peroxidase conjugate. In heterozygous (Dlb-1^a/ Dlb-1^b) mice, loss of the Dlb-1^b allele by mutation, results in a ribbon of non-staining cells, extending up a villus of the small intestine (Winton et al., 1988). These mutations arise in stem cells located at the base of crypts that feed cells to the villus. The *Dlb-1* gene has been mapped to mouse chromosome 11 (Uiterdijk et al., 1986), but has yet to be cloned.

1.7 TRANSGENIC MOUSE ASSAYS

It is evident that the characteristics of a genetic assay have an impact on the ability and the extent to which the system can detect and quantitate mutations. The recent development of transgenic mice has introduced new possibilities in the field of mutation research by overcoming some of the obstacles posed by *in vivo* assays. Tissue-specific mutation induction, *in situ* metabolic activation, DNA sequence alteration and germ cell mutagenesis can now be studied *in vivo* using one mutation assay (Suzuki et al., 1994).

Currently, two transgenic mouse systems suitable for mutation analysis, are commercially available, Big Blue™ and Muta™ Mouse. Each mouse has multiple copies of lambda shuttle vectors containing either the bacterial *LacI* (Big™ Blue) or *LacZ* (Muta™ Mouse) gene as mutational targets, stably integrated within its genome in every cell (Kohler et al., 1990; Gossen et al., 1989).

1.7.1 *LacI* Transgenic Mouse Assay

The *LacI*/Big Blue™ transgenic mouse system was developed by microinjecting a bacteriophage lambda shuttle vector containing a full length bacterial *lacI* target gene, *lac*O gene and the α*LacZ* reporter gene, into inbred C57Bl/6 mice (Kohler et al., 1990). The *lacI* gene encodes a repressor protein for the bacterial lactose (lac) operon. If the gene is not mutated, repressor protein binds to the operator sequence and prevents transcription of the downstream *LacZ* gene. Should there be a mutation within the *LacI* gene, the repressor protein is unable to bind and the *LacZ* gene is consequently transcribed forming functional β-galactosidase enzyme. Phage-infected bacteria are plated on agar containing the chromogenic substrate X-gal. Cleavage of X-gal by β-galactosidase will form blue plaques while nonmutant *lacI* genes will produce colourless plaques. Mutant frequency is then determined by the ratio of colourless to colored plaques.

The complete lambda ZAP/*lacI* shuttle vector is approximately 46 kb in length and is integrated in 40 tandem copies into chromosome 4 (Dycaico et al., 1994). The *lacI* gene itself is small, 1080 base pairs and therefore suitable for sequencing. Since genomic DNA can be isolated from any tissue of interest, comparative mutation analysis between tissues is possible.

1.7.2 *LacZ* Transgenic Mouse Assay

The *LacZ*/Muta™ Mouse transgenic system is similar to Big Blue™ except for two notable differences; the bacterial *lacZ* gene serves as the mutational target and the detection of mutants is based on a positive selection system (Gossen et al., 1992; Gossen and Vijg, 1993). Forty copies of the *lacZ*/λgt10 shuttle vector construct have been integrated as a head-to-tail concatamer on chromosome 3 of CD-2 (BALB/c x DBA) mice (Swiger et al., 1994; Blakely et al., 1995). The entire cassette is approximately 47 kb in length with the *lacZ* gene occupying 3,126 base pairs (Myhr, 1991). Since the nucleotide sequence of the *lacZ* gene is known, mutations can also be characterized at the molecular level.

The *E. coli* strain used for mutant selection is nonfunctional at the galactose-4-epimerase (*galE*) locus and is therefore, unable to convert UDP-galactose into UDP-glucose (Gossen et al., 1992; Gossen and Vijg, 1993). A nonmutant *lacZ* gene will produce a functional β-galactosidase protein, which cleaves the lactose analog, phenyl-β-*D*-galactopyranoside (p-gal) into phenol and galactose. The pathway stops however and

bacterial lysis occurs due to the toxic effect of the accumulation of UDP-galactose. No plaque will develop as a result. In contrast, mutant lacZ genes produce a plaque since the breakdown of p-gal does not occur. Mutant frequency is determined by the ratio of plaques that grow in the absence of p-gal (non-selective agar) to the number of plaques that grow in its presence.

The size of DNA that can be packaged into phage heads is restricted to a 45 to 55 kb size range. As a result, the types of mutations that can be detected with the Muta™ Mouse and Big Blue™ systems is limited to point mutations and small deletions (less than 4 kb) and insertions (less than 6 kb) (Gossen et al., 1989; Kohler et al., 1991; Provost et al., 1993).

Since the Muta™ Mouse transgenic system allows for the study of mutagenesis in any tissue, the mouse small intestine and colon have been selected for study, primarily for one reason. Colon cancer is the third most common malignancy in the Western world. The most important environmental cause of colorectal cancer is diet. A high fat and low vegetable and fiber intake is associated with an increased risk for this cancer (Kritchevsky, 1993; Harris and Ferguson, 1993; Witte et al., 1996). The mouse small intestine and colon provide representative models for studying the effect of diet on mutagenesis in relation to carcinogenesis of these tissues.

1.8 THE MOUSE SMALL INTESTINE AND COLON AS A MODEL FOR MUTAGENESIS

The mammalian small intestine has a single layer of epithelial cells organized into villi (finger-like projections) and crypts (tubular invaginations surrounding the villi) (Potten and Loeffler, 1990). The cells of the intestine are in a constant state of flux; new cells are continuously being produced within the crypt, while cells that have migrated up to the villi, are eventually shed into the intestinal lumen. The entire epithelial layer is replaced every 5-7 days. In the adult mouse, there are approximately 100,000 villi making up the small intestine. Each is supplied by on average, 10 crypts (Cosentino et al., 1996).

Stem cells, located at the base of each crypt, maintain the cell population of the crypt and villus. They divide to produce cells that make up the proliferation zone of the crypt. The cells of this region and their progeny undergo further mitotic division and subsequently migrate up into the maturation zone, where cells assume a functional identity. Mitotic activity has ceased but constant pressure from below continues to move cells up the crypt and onto the villus. Each crypt contains approximately 250 cells, one of which is a stem cell (Potten and Loeffler, 1990; Cosentino et al., 1996).

It is evident that if a mutation arises in the stem cell, all of the subsequent progeny will also be mutated (Winton et al., 1988, 1990, 1991). Since each villus is supplied by a number of crypts, a ribbon of mutated cells arises along the epithelium. This provides the basis for the *Dlb-1* assay discussed earlier. Although mutations may arise in any cell of

the crypt, only those that occur in a stem cell are maintained since these cells are the only permanent residents of the crypt.

The colon is organized similarly to the small intestine except for a lack of villi and a cell turnover time about 2 days longer than that of the small intestine (Dawod et al., unpublished data). Cells migrate from each colonic crypt to cuff of surface epithelial cells that surround the opening of each crypt. Each crypt is maintained by a single, multipotent stem cell and proliferative capacity of colonic crypts is comparable to those of the small intestine (Gordon et al., 1992). Mutant frequencies in the colon have been found to be similar to those measured in the small intestine based on data accumulated in this laboratory.

1.9 OUTLINE OF THESIS

While it is widely accepted that DNA damage and somatic mutations are important in carcinogenesis, the influence of diet on spontaneous genetic events has received limited study. Utilizing two *in vivo* genetic assays, the micronucleus assay and the Muta™ Mouse mutation system, this work was undertaken to examine the hypothesis that the correlation between diet and cancer is the result of an effect of nutritional factors on genetic damage and the resulting somatic mutation frequencies. Furthermore, it was meant to provide an alternative approach to epidemiological studies and animal experiments with carcinogens for identifying dietary foods or constituents most effective at protecting against DNA damage. In previous work by Paashius-Lew and Heddle,

1997, it was shown that most spontaneous mutations arise during early development. Since vegetables, fruits and grains are known to contain anti-mutagens and anti-carcinogens, then increased intake of these foods during pregnancy and lactation may reduce mutation rates and risk to cancer later in life.

The first study examined the effect of maternal folate levels on micronucleus and *lacZ* mutation frequencies in the developing animal. Folic acid is essential for genetic and cellular integrity and inadequate folate levels have been shown to induce chromosome damage in adult rodents and humans. Since folic acid is required for DNA replication, low concentrations of maternal folate during pregnancy may disrupt DNA synthesis in fetal development and result in an elevated mutation frequency in the offspring. Conversely, folic acid supplements during pregnancy could be beneficial in reducing the mutation rate observed during this period. No differences in mutant frequencies at the *lacZ* transgene were found between offspring whose mothers were fed low folate diets and those on high folate diets during pregnancy. Similar results were observed with respect to micronucleus frequencies. These findings suggest that the minimum recommended requirement of folic acid may be sufficient for limiting genetic damage and additional supplementation is of no greater benefit.

There are a large number of dietary foods that could be analyzed for their effects on somatic mutations but the time and expense involved in measuring mutations with transgenic assays do not permit testing them all. The second part of this project consisted of screening a number of vegetables, fruits and grains for their effect on spontaneous

cytogenetic damage using the peripheral blood micronucleus assay. Since spontaneous events are rare, automated methods for measuring micronuclei were used, increasing the reliability and accuracy of the results. Of the 26 foods tested, dietary supplementation of flaxseed was found to significantly reduce chromosome damage in erythrocytes of mice over 44 days, demonstrating an anti-mutagenic property for flaxseed.

In the last chapter, several foods used in the micronucleus study were examined for their effects on spontaneous *lacZ* mutations during development. Mutation frequencies in the colon of mice fed supplemented diets did not differ from control (AIN-93G) frequencies indicating the lack of any protective effect by these foods. Comparison with previous data from mice fed laboratory rodent chow however, indicated a significantly elevated mutant frequency as compared to control frequencies observed in this study. The AIN-93G diet is nutritionally better than laboratory chow. The results suggest that nutritional deficiencies in the standard diet could increase the mutation rate and consequently, may increase the rate of cancer.

FIGURE LEGENDS

Figure 1. Spontaneous mutant frequencies as a function of age. Data replotted from Ono et al., (1995) and Paashuis-Lew and Heddle, (1997). The mutant frequency at conception is 0, since inherited mutations are evident as high mutant frequencies ("jackpots").

Figure 2. Mutant frequency as a function of the number of cell divisions (replotted from Heddle et al., 1996). Assuming a constant mutation rate per division, the accumulation of mutants is linear.

Figure 3. Mutant frequency as a function of the number of cells (replotted from Heddle et al., 1996). The number of cells can be taken as a substitute for weight or age.

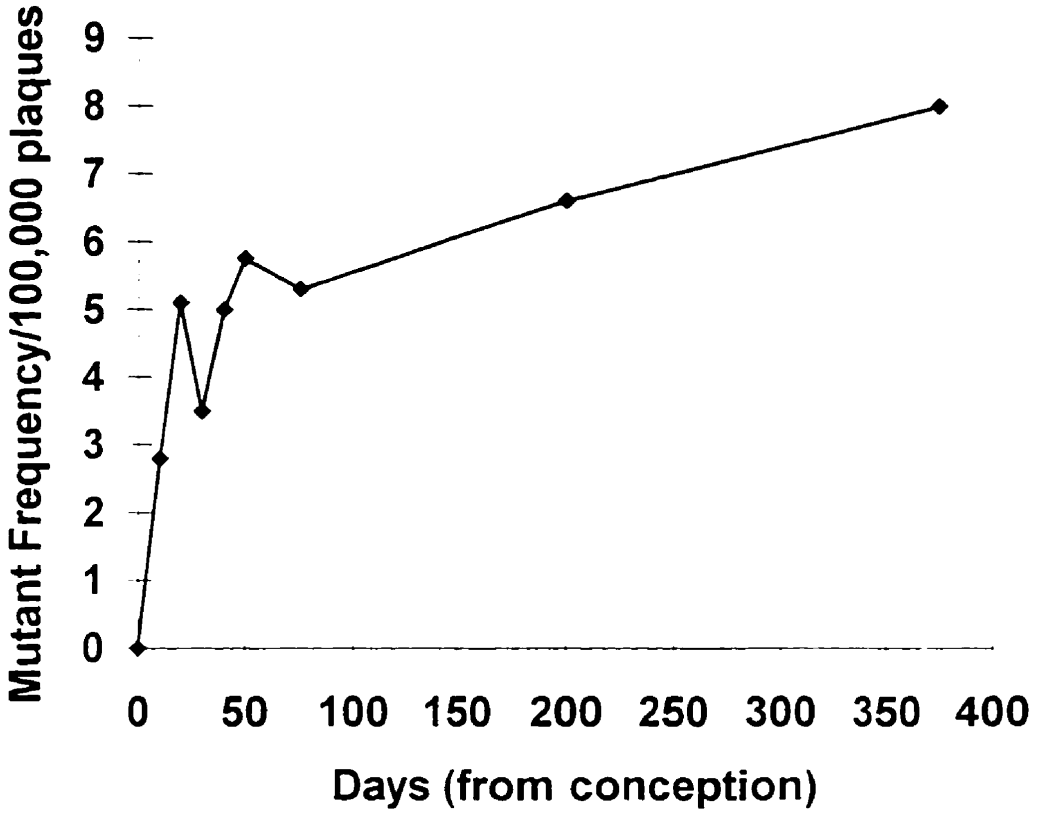
Mutant Frequency/100,000 plaques
9
8
7
6
5
4
3
2
1
0
0
50
100
150
200
250
300
350
400
Days (from conception)

FIGURE 1

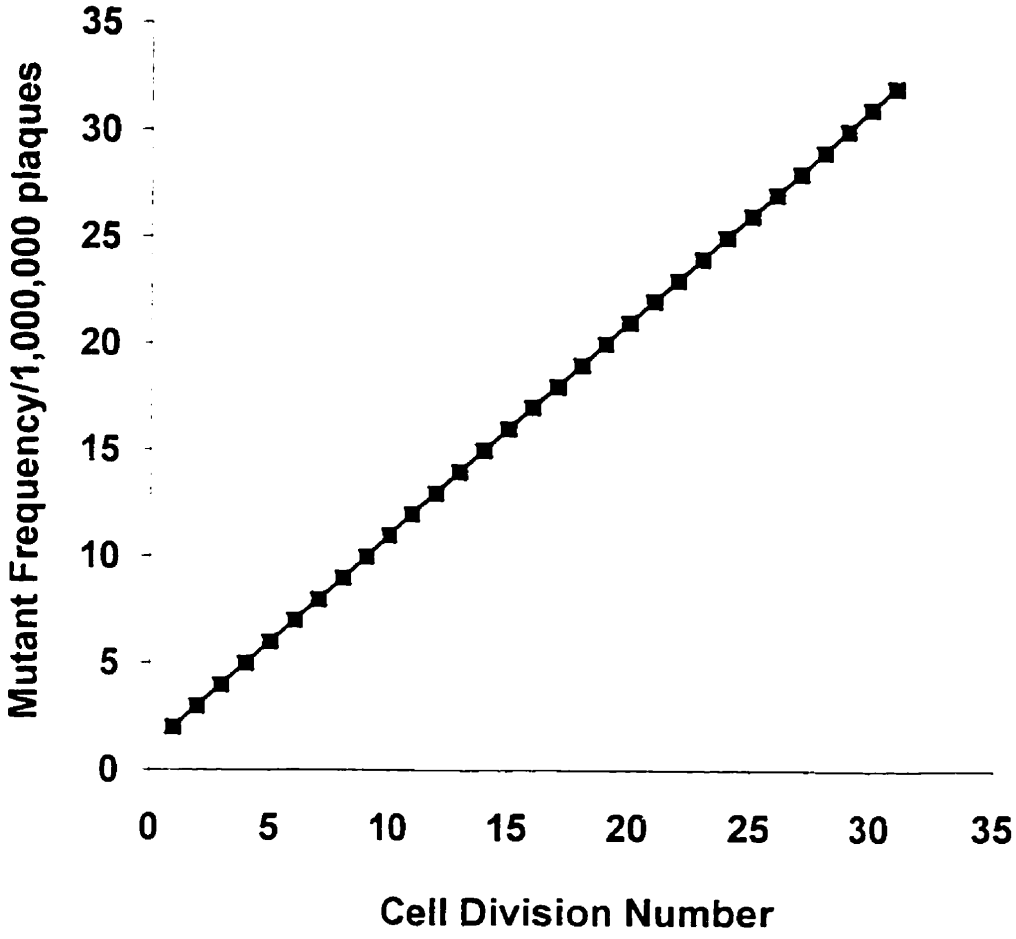
Mutant Frequency/1,000,000 plaques
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
35
Cell Division Number

FIGURE 2

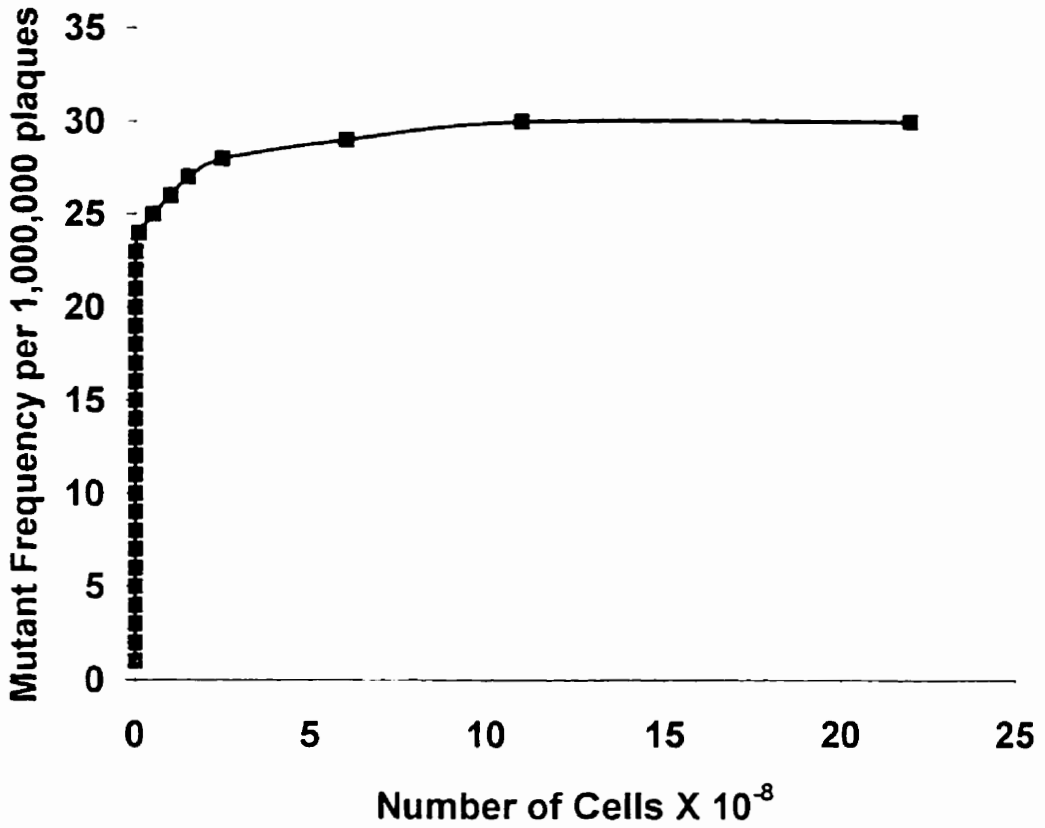
Mutant Frequency per 1,000,000 plaques
35
30
25
20
15
10
5
0
0
5
10
15
20
25
Number of Cells X 10-8

FIGURE 3

CHAPTER 2

EFFECT OF MATERNAL FOLATE LEVELS ON SOMATIC MUTATION FREQUENCY DURING DEVELOPMENT

(folic acid, development, *lacZ*, micronuclei, *Dlb-1*)

by

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2.1 INTRODUCTION

Folic acid is among a number of dietary constituents considered to be possible modulators of carcinogenesis. Epidemiological evidence has suggested a significant association between higher folate intake and lower risk of several epithelial cancers including the colon (Cravo et al., 1992; Freudenheim et al., 1991). The biosynthesis of nucleotides, particularly thymine, and the metabolic cycle that maintains the supply of *S*-adenosylmethionine for methylation reactions are absolutely dependent on adequate concentrations of folic acid (Jennings, 1995). Folate deficiency in both animal and *in vitro* studies, is associated with an increase in micronuclei and chromosomal aberrations including chromosome breaks, defects in condensation and expression of fragile sites (Reidy et al., 1983; Everson et al., 1988; MacGregor et al., 1990). This effect is a consequence of alterations in the pools of nucleotides required for DNA synthesis (MacGregor, 1990).

Mutations have a critical role in cancer onset (Vogelstein et al., 1989; Vogelstein and Kinzler, 1993). Tumors arise as a result of the accumulation of genetic damage. For most cancers, somatic rather than inherited mutations play a more important role. A fraction of these mutations are unavoidable arising as a consequence of normal cellular functions. Recently, it has been found that many of these spontaneous mutations arise early in life, during embryogenesis and fetal growth, with a lower rate of accumulation later on. At the *hprt* locus in humans, approximately 2/3 of all mutants acquired over a lifetime occur by 20 years of age (Finette et al., 1994; Robinson et al, 1994). Similar

results were observed in two transgenic mouse systems (Lee et al., 1994; Ono et al., 1995; Paashius-Lew and Heddle, 1997). This pattern has been associated with the rate of cell division (Heddle et al., 1996; Paashius-Lew and Heddle, 1997). Early development is characterized by a high rate of cell proliferation that is unlike any other period in life. Proliferation is an important factor in mutagenesis and carcinogenesis (Ames and Gold, 1990). Increased cell division increases the likelihood of DNA replication errors and DNA attack by endogenous and exogenous agents (Ames and Gold, 1991).

Folic acid deficiency is highly prevalent in the Western world, especially among pregnant women (Zitay, 1969; Eto and Krumdieck, 1986). Folate deficiency during pregnancy is associated with an elevated incidence of birth defects (Milunsky et al., 1989). Since cellular nucleotide levels are maintained by folic acid, increased sensitivity of the developing fetus to mutations arising from replication errors is probable under conditions of low maternal folate. A consequence of an elevated mutant frequency is an increased risk of cancer in later life. Altering the diets of the mother and infant to include vitamin supplements may reduce the frequency of somatic mutations and the resulting cancers.

To test this hypothesis, the effect of high and low maternal folate levels on mutation frequencies during development was examined. Mutant frequencies in the epithelial cells of mouse small intestine were measured using the Muta™ Mouse transgenic system and the endogenous *Dlb-1* gene. Additional mutation measurements at the *lacZ* transgene were made in the colon. Since an elevated frequency of

micronucleated erythrocytes is associated with folate deficiency, the micronucleus assay was also used to quantify cytogenetic damage in peripheral blood.

2.2 MATERIALS AND METHODS

Animals and Diets. The experiments reported here were approved in advance by the animal care committee of York University and conformed to the Canadian guidelines for animal care.

All mice used in the experiments were the F_1 offspring of SWR females (*Dlb-1*^{a}/*Dlb-1*^{a}, Jackson Laboratories, Bar Harbor ME) and Muta™ Mouse males (*Dlb-1*^{b}/*Dlb-1*^{b}, Hazelton Research, VA). All F_1 offspring were hemizygous for the *lacZ* transgene and heterogygous for the *Dlb-1* locus, namely *Dlb-1*^{a}/*Dlb-1*^{b}. Folic acid-deficient AIN-93G diet, folic acid and glycine were purchased from ICN Biomedicals (Aurora, OH). The antibiotic succinylsulfathiazole was obtained from Sigma (St. Louis, MO).

The mice were housed in stainless steel wire-bottomed cages at 70 % humidity and a temperature of 22±2 °C with a 12 h light/dark cycle. Diet and water were fed *ad libitum*. Diets were prepared by adding 25 g of glycine and 10 g of succinylsulfathiazole per kilogram of diet to folic acid-deficient AIN-93G diet. Since intestinal bacteria produce

folate, in order to eliminate the supplementation of folic acid via copraphagy, the sulfa antibiotic is commonly added to the diet. Excess glycine is added to potentiate the deficiency. Folic acid was mixed in at a concentration of 0.5, 1, 2, 20 or 200 milligrams per kilogram of diet. A minimal volume of distilled water was used in order to pellet the food. Diets were subsequently stored at 4 °C until use.

The SWR females were randomly assigned to and fed one of the five experimental diets for 35 days prior to initiating breeding. Three SWR females were bred with one Muta™ Mouse male for each treatment group. The male mouse was removed once a visible sign of pregnancy was observed and each pregnant female was then housed individually. The females continued on the assigned diets for the entire duration of pregnancy and lactation. Half of the offspring of each dam was sacrificed at 3 weeks of age for mutation analysis and the remainder continued on the diets assigned until 8 weeks of age, at which time they were sacrificed. The number and gender of the pups analyzed at 3 and 8 weeks depended upon the gender composition and litter size in each treatment group.

The *LacZ* Transgenic Mouse Assay. Genomic DNA was extracted from both small intestinal and colonic epithelial cell suspensions. After sacrifice, the entire small intestine was removed from the mesentery and the upper and lower thirds of the intestine were used for DNA extraction. Each section was flushed with phosphate buffered saline (PBS; pH 8.0) and inverted. The tissue was then placed in 3 ml of 75 mM KCl/20 mM

EDTA and gently forced in and out of a 5 cc needless syringe to remove the epithelial cells from the intestine. The suspension was collected into a 15 ml centrifuge tube, frozen in liquid nitrogen and stored at -70 °C until the time of extraction.

Similarly, the entire colon was also removed at sacrifice, flushed with PBS, inverted and placed in either 2 ml of 75 mM KCl/20 mM EDTA solution and gently forced in and out of a 5 cc needless syringe. The cell suspension was again collected, frozen in liquid nitrogen and stored at -70 °C.

To extract the DNA, the cells were digested with 5 ml of lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 20 mM EDTA) containing 0.5 ml of 10% SDS, 20 mg/ml proteinase K (ICN Biomedicals, Aurora, OH) and 100 μg/ml RNase A (ICN Biomedicals) in a 55 °C water bath for 3 hours. The DNA was then extracted from the digested samples using 2 phenol:chloroform (1:1) extractions followed by one chloroform extraction. After each extraction, the tubes were spun in a bench top centrifuge at 1000 x g for 15 minutes and the top aqueous layer containing the DNA was transferred to a sterile polypropylene centrifuge tube. The DNA was precipitated with 2 volumes of 100% and spooled out of the tube with a micropipet that had been flamed to a hooked-shape. The DNA was dried and then dissolved in TE buffer (10 mM Tris-HCl, pH 7.5; 1.0 mM EDTA, pH 7.5). DNA concentrations used for packaging were between 0.5 and 5.0 μg/μl.

The lambda phage shuttle vector, which contains the entire the entire *lacZ* mutational target gene, was recovered by *in vitro* packaging with Mutaplax™ packaging extract (Epicentre Technologies Corp., Madison, WI). 5 µl of DNA was added to 25 µl of packaging extract and incubated for 1.5 hours at 30 °C. An additional 25 µl was then added and the reaction incubated for a further 1.5 hours. 450 µl of phage buffer (10 mM Tris, pH 8.3; 100 mM NaCl; 10 mM $MgSO_4$) was added to stop the reaction and allowed to sit at room temperature for 30 minutes. 1 ml of overnight culture of *E.coli* C (amp^r, kan^r, lac-, galE-, recA-) was incubated at 37 °C for 3-4 hours in LB broth containing 1 % maltose and 10 mM $MgSO_4$ to a spectrophotometer reading between OD_{600}=0.5 to OD_{600}=1.0. The culture was spun down in a bench top centrifuge for 10 minutes at 1000 x g and resuspended in fresh LB media containing 10 mM $MgSO_4$. 500 µl of the packaged DNA was added to 2 ml of *E.coli* culture and incubated for 20-30 minutes at room temperature. Phage-infected *E.coli* was then plated on an LB plate (8 ml diluted to 25 %; 15 gm/l agar) with 10 ml LB (diluted to 25 %; 7.5 gm/l agar, 10 mM $MgSO_4$) supplemented with 0.003 % phenyl β-*D*-galactopyranoside (p-gal; Sigma, St. Louis, MO) as a selection agent. When the lambda DNA is excised, packaged into phage heads, and adsorbed into *E.coli* (lac^-), the *lacZ* gene is constitutively transcribed and produces β-*D*-galactosidase. Mutated *lacZ* genes produce no functional enzyme. When lambda phage with a mutant *lacZ* gene are plated in the presence of a lactose analog such as p-gal, a plaque forms. If the *lacZ* gene is functional however, a plaque formation is prevented

because cleavage of the β-*D*-galactoside bond results in a toxic accumulation of UDP-galactose due to a *galE* mutation in the *E.coli*. Thus, only mutants can develop plaques in the presence of p-gal. To determine the phage titre for the reaction mixture and thus determine the number of loci analyzed, 5 μl of adsorbed *E.coli* was diluted in 100 μl of LB media containing 1μl of $MgSO_4$. 20 μl aliquot was removed and added to 1 ml of *E.coli* culture. 12 ml of LB was subsequently added and the entire bacteria/phage mixture was poured onto 2 LB plates. Titre plates were grown under non-selective conditions to allow for growth of nonmutant phage. All plates were incubated overnight at 37 °C.

The *Dlb-1* Locus Assay. Whole mounts of 3 and 8 week small intestine were prepared as described in Winton et al., (1988) and Schmidt et al., (1990), with a few modifications (Tao et al., 1993). The middle third of the small intestine (jejunum-ileum) was flushed with PBS. One end of the intestine was held closed by microscope slides fastened by a clip. The intestine was inflated with 10 % formal saline (0.85 % NaCl, in 10 % formalin, Sigma, St. Louis, MO) and allowed to fix for 3 minutes. The small intestine was then drained and cut along the mesenteric line and laid flat on a microscope slide, and held in place with plastic coated paper clips. The slides were fixed for at least 1 hour in 10 % formal saline, rinsed with PBS and incubated in 20 mM DL-dithiothreitol (Sigma, St. Louis, MO) in 20 % ethanol and 80 % Tris-HCl (pH 8.0) for a minimum of 45 minutes, to remove the mucus. The slides were then stored in 10 % formal saline until staining.

At the time of staining, the slides were incubated in 0.1 % phenylhydrazine-HCl (Sigma, St. Louis, MO) in PBS for 30 minutes to block endogenous peroxidases, followed by a 20 minute incubation in 0.5 % bovine serum albumin (BSA; Boehringer, Laval, PQ) in PBS, and subsequently left overnight at room temperature in 5 µg/ml *Dolichos biflorus* agglutinin-peroxidase conjugate (Sigma, St. Louis, MO) dissolved in PBS-BSA solution. The following day, all slides were rinsed twice with PBS, once with PBS-BSA and then incubated in 0.5 mg/ml 3,3'-diaminobenzidine (Sigma, St. Louis, MO) dissolved in Tris-HCl (pH 4.0) with 250 µl hydrogen peroxide for 45 minutes or until sufficiently brown. The slides were rinsed twice with PBS and then stored in 10 % formal saline until scoring.

Each slide was coded and scored using a dissecting microscope at a magnification of 50 X. The *Dlb-1*^{b}/*Dlb-1*^{a} epithelial cells appear as brown stained cells, whereas mutant cells (*Dlb-1*$^{-}$/*Dlb-1*^{a}) do not stain and appear as vertical white ribbons on the villi. The first and last field, defined by a rectangle in an eyepiece graticule, of every slide was counted twice and the counts averaged to estimate the number of villi per field. The total number of fields scored and the number of mutants observed for each slide were noted.

Peripheral Blood Micronucleus Assay. In order to determine micronucleus frequencies in 3 and 8 week-old pups, bloodsmears were made from a sample of peripheral blood at the time of sacrifice. The slides were left overnight to dry and then

fixed in 100% methanol for 20 minutes. The slides were then stored at room temperature until staining. Bloodsmears were stained with 0.125 mg/ml acridine orange (ICN, Aurora, OH) in M/15 Sorensen's buffer (pH 6.4; 70 mL M/15 KH_2PO_4, 30 mL M/15 Na_2HPO_4) for 1 minute. The slides were then rinsed in Sorenson's buffer and incubated in fresh buffer for 15 minutes. A second incubation in fresh buffer for 15 minutes followed and the slides were then stored dry in a light tight slide box until scored.

Each slide was coded and scored using a fluorescence microscope at a magnification of 100 X. Wet mounts were made by dipping the slides in Sorenson's buffer and overlaying a coverslip. Acridine orange staining allows for discrimination of DNA from RNA as the latter emits a red fluorescence compared to the former, which fluoresce bright yellow. Immature erythrocytes or reticulocytes can be therefore distinguished from mature erythrocytes by their bright red staining due to the presence of RNA. Since mature erythrocytes lack nuclei, these cells appear dull green. Micronuclei were defined as intracellular bodies with the characteristic yellow fluorescence of DNA. The numbers of micronucleus-containing cells among at least 1,000 reticulocytes and 10,000 mature erythrocytes were determined for each slide based on methods described in Smith et al., 1990 with some modifications. The total number of reticulocytes scored was determined by counting the number of reticulocytes in every field scored. The total number of erythrocytes scored was determined by counting the number of erythrocytes in an area that was approximately one-half the area of every 10^{th} field. The total number of fields

and the total number of micronucleated reticulocytes and micronucleated erythrocytes scored were noted.

Statistics. Statistical analyses were conducted with Microsoft Excel software. The tests used were mainly one-way anovas, 2-sample t-tests and regression analyses. All means and standard errors reported are based on the mutant frequency observed in each animal, regardless of the number of plaques, villi or micronuclei analyzed.

2.3 RESULTS

All mutation data collected for 3 and 8 week-old offspring using the micronucleus assay, Muta™ Mouse transgenic system and *Dlb-1* locus are shown in Tables 1 to 7 of Appendix A. Litter sizes are within normal ranges for most dams in the 0.5, 2 and 200 mg/kg treatment groups. Dams with no surviving offspring following two attempts at breeding were eliminated from the study. For this reason, the data are limited for the 1 and 20 mg/kg groups. The reproductive period of female SWR mice lies within the first 6 months after birth. Many of the females were 5 months of age at the time of breeding and their reproduction capacity may have already been reduced. Interpretation of data will therefore focus primarily on the 0.5, 2.0 and 200 mg/kg groups. Low levels of folic

acid had no effect on dam or pup weights during the course of the study. Mice on low levels weighed as much as those fed higher levels.

The frequencies of micronucleated reticulocytes and mature erythrocytes in 3 and 8 week-old mice as a function of folic acid concentration are shown in Figures 1 and 2, respectively. The presence of micronuclei in reticulocytes is an indication of recent damage as reticulocytes represent the immature erythrocyte cell population of the blood. No significant change in reticulocyte micronucleus frequencies was observed with increasing folate concentration for either the 3 or 8 week age groups (F_3=0.41; DF=2, 12; P=0.67; F_8=0.84; DF=2, 13; P=0.45). The stability in micronucleus frequency over folic acid concentrations is more evident in the mature erythrocytes of both age groups. Again, no significant difference in mean frequencies is found (F_3=0.80; DF=2, 12; P=0.47; F_8=3.23; DF=2, 13; P=0.07). Regression analysis of micronucleated reticulocyte and mature erythrocyte data did however indicate a slight decrease in frequencies with increasing concentrations of folate.

The spontaneous *lacZ* mutant frequencies in 3 and 8 week small intestine are shown in Figure 3. While the data appears to show a mutagenic effect of folate supplementation, the association is not statistically significant. Mice maintained on a high-folate diet exhibited no significant difference in frequency of *lacZ* mutations compared to mice on low folate diets (F_3=2.52; DF=2, 20; P=0.11; F_8=0.40; DF=2, 9; P=0.68). This is also true for mutant frequencies observed in the colon (Figure 4; F_3=0.16; DF=2, 20; P=0.86; F_8=0.07; DF=2, 17; P=0.94) and at *Dlb-1* locus (Figure 5;

F=1.78; DF=2, 16; P=0.20). Although the data were not significant, regression analysis indicated a linear increase in mutations with increasing folate concentrations for all cases.

No significant differences were found when each set of 3 week data was compared with its 8 week counterpart. In addition, mutant frequencies of pups from one dam were found not to be statistically significant from pups of another dam in the same treatment group for all data analyzed.

2.4 DISCUSSION

The accumulation of genetic damage in developing mice under conditions of low and high maternal folate levels was measured using three *in vivo* mutation assays. Although previous studies with the micronucleus assay have shown an elevated frequency of chromosomal aberrations with folate deficiency (Everson et al., 1988; MacGregor et al., 1990), no significant differences in micronucleus and *lacZ* and *Dlb-1* mutation frequencies were found between pups fed diets containing 0.5 mg/kg folic acid and those fed diets with 200 mg/kg folic acid. This lack of mutagenicity is not entirely unexpected however. No study has examined the mutational effects of very low concentrations of the vitamin in developing animals. It is possible with adult rodents to use diets completely void of folic acid. Successful reproduction however, requires a minimum dietary concentration of 0.5 mg/kg (Heid et al., 1992). Inadequate folate levels results in smaller sized litters with lower birth weights and poor survival rate. The

National Research Council (1978) has defined the minimum daily requirement of folic acid for normal growth of mice to be 0.5 mg/kg. This minimum requirement may therefore be adequate to have no significant effect on nucleotide pools and thus mutation frequencies, as compared to diets completely deficient of folate. Furthermore, the role of folic acid in reducing the incidence of certain cancers may be similar to other vitamins where the minimum requirement is sufficient and any higher consumption is not of greater benefit (Ames, Gold and Willett, 1995). The absence of any effect on dam and offspring body weights may provide some support for this possibility. In previous studies, mice fed folate-deficient diets weighed 20 % less that those on folate-replete diets several weeks after the initiation of experiments (Bills et al., 1992a; Heid et al., 1992).

Studies examining the role of folic acid supplementation on carcinogenesis in laboratory animals have been limited to effects on carcinogen-induced tumors, particularly in the colon. Results from these studies have been inconsistent. Folate deficiency was found to enhance the development of colonic neoplasia induced by 1,2-dimethylhydrazine (Cravo et al., 1992). Rats fed diets containing 8 mg/kg folic acid in the same study were free of any neoplastic lesions. In contrast, folate deficiency suppressed, and folate supplementation (20 and 40 mg/kg) enhanced, the incidence of rat mammary tumors induced by methylnitrosurea (Baggott et al., 1992). An additional study found that the onset of spontaneous tumors in *tax1* transgenic mice could be delayed by feeding mice the minimal dietary requirement of folic acid (Bills et al.,

1992b). The association of folate supplementation with enhanced cancer induction has been suggested to be an effect of a necessity of folic acid for cancer growth (Baggott et al., 1992). The progression of a pre-malignant cell to a malignant tumor involves excessive cell proliferation. A balance in the nucleotide pools required for DNA replication is dependent on the availability of folic acid.

Surprisingly, although *lacZ* mutation frequencies in the small intestine and colon were not statistically different between groups, regression analysis indicated an increase in frequencies with increasing folic acid concentration. Likewise for *Dlb-1* mutations. If low folate decreases the pool of thymine, high levels may elevate it increasing the likelihood of misincorporation of thymine. The imbalance is likely not as severe as with folate deficiency otherwise regression analysis would not have indicated a slight decrease in micronucleus frequencies. Repair is efficient to correct misincorporations without producing chromosome breakage.

Since the National Research Council has defined the maximum daily requirement of folic acid for mice to be 2.8 g/kg of diet, it would be of interest to examine the mutational effects of folate concentrations in excess of 200 mg/kg. Higher levels may prove to be more harmful than beneficial. Additional mutation studies with folic acid supplements would be necessary in order to establish any role of folate in limiting cancer incidence.

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FIGURE LEGENDS

Figure 1. Frequency of micronucleated reticulocytes in the peripheral blood of 3 and 8 week-old mice as a function of folic acid concentration. Values are means ± SEM.

Figure 2. Frequency of micronucleated mature erythcytes (NCE) in the peripheral blood of 3 and 8 week-old mice as a function of folic acid concentration. Values are means ± SEM.

Figure 3. Spontaneous *lacZ* mutant frequencies in the small intestine of 3 and 8 week-old mice as function of folic acid concentration. Values are means ± SEM.

Figure 4. Spontaneous *lacZ* mutant frequencies in the colon of 3 and 8 week-old mice as a function of folic acid concentration. Values are means ± SEM.

Figure 5. Spontaneous mutant frequencies at the *Dlb-1* locus of 8 week-old mice as a function of folic acid concentration. Values are means ± SEM.

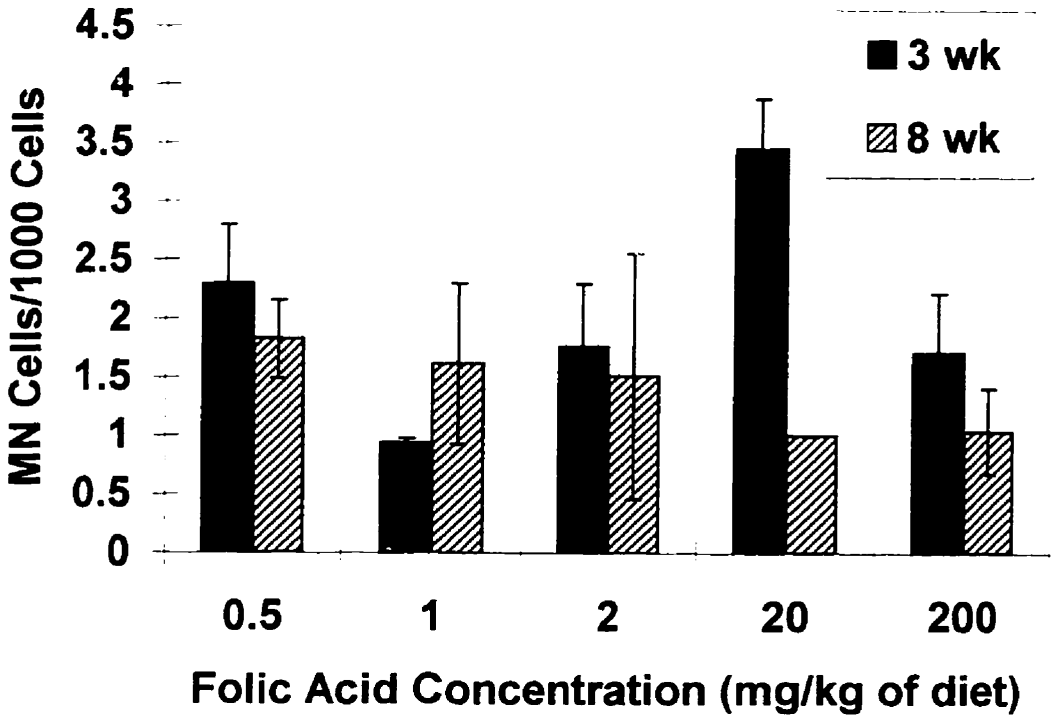
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
MN Cells/1000 Cells
3 wk
8 wk
0.5
1
2
20
200
Folic Acid Concentration (mg/kg of diet)

FIGURE 1

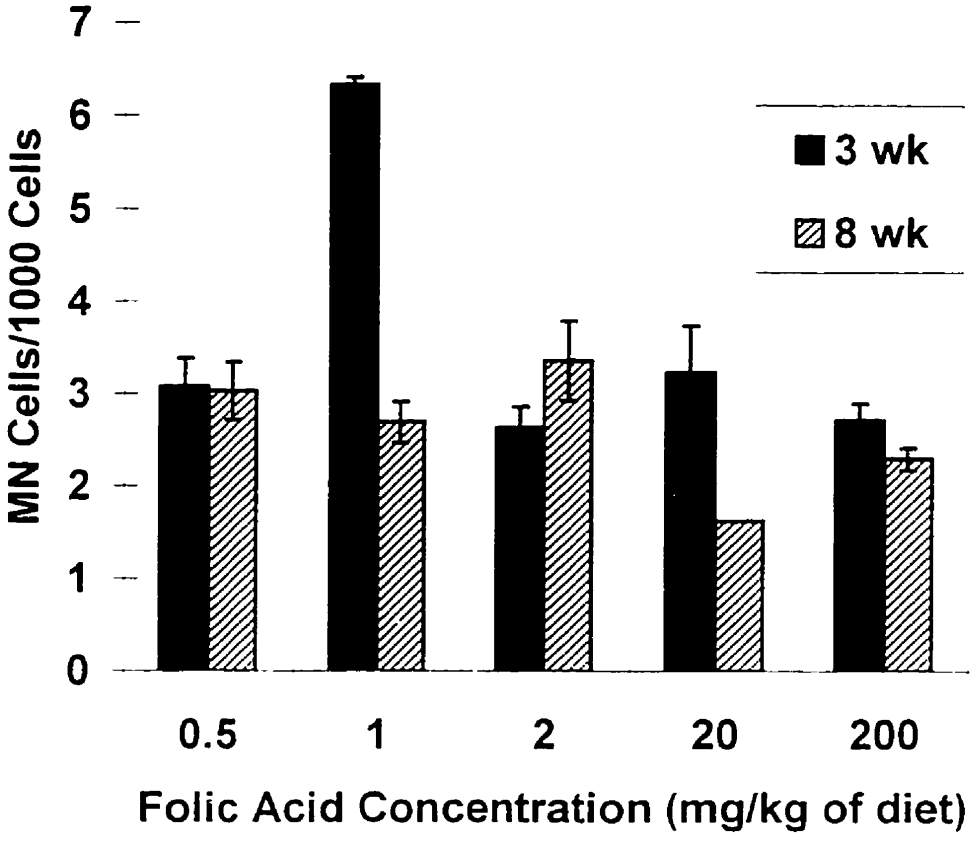
MN Cells/1000 Cells
7
6
5
4
3
2
1
0
3 wk
8 wk
0.5
1
2
20
200
Folic Acid Concentration (mg/kg of diet)

FIGURE 2

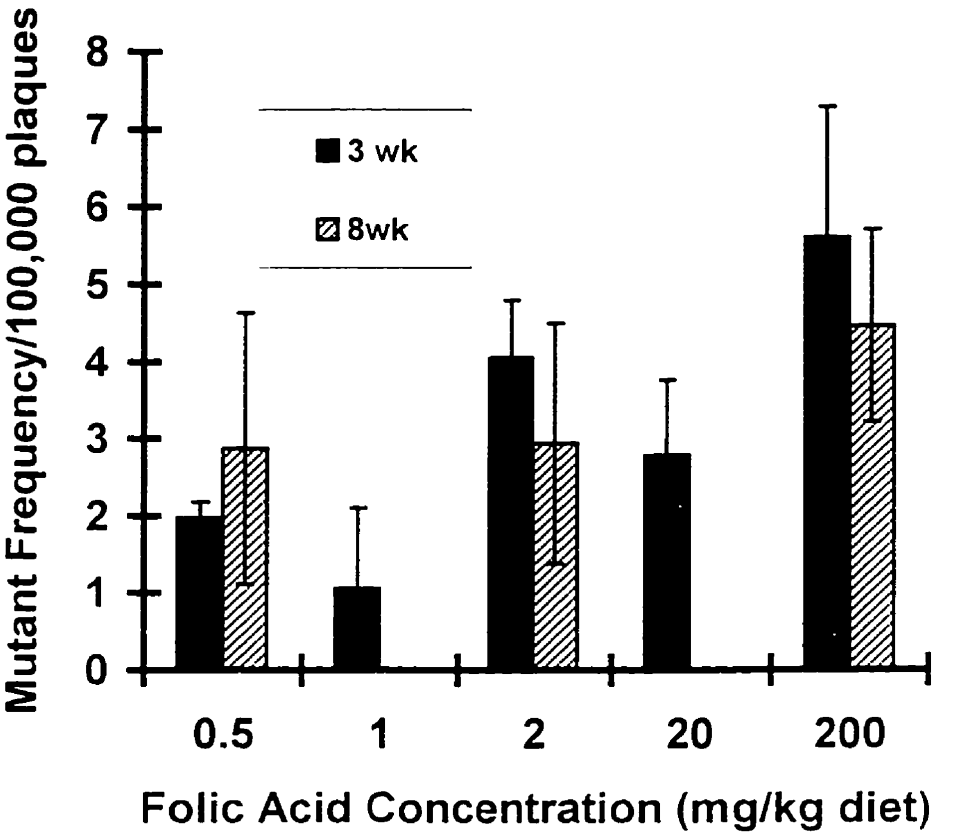
Mutant Frequency/100,000 plaques
8
7
6
5
4
3
2
1
0
3 wk
8wk
0.5
1
2
20
200
Folic Acid Concentration (mg/kg diet)

FIGURE 3

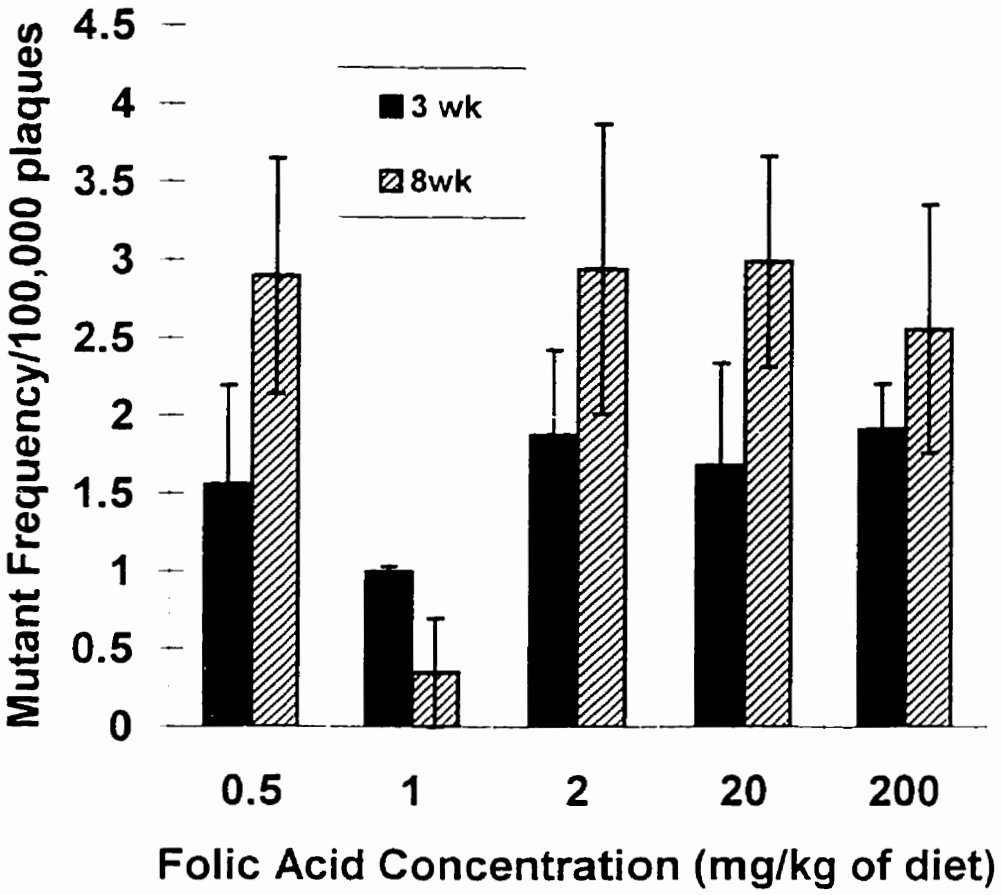
Mutant Frequency/100,000 plaques
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
3 wk
8wk
0.5
1
2
20
200
Folic Acid Concentration (mg/kg of diet)

FIGURE 4

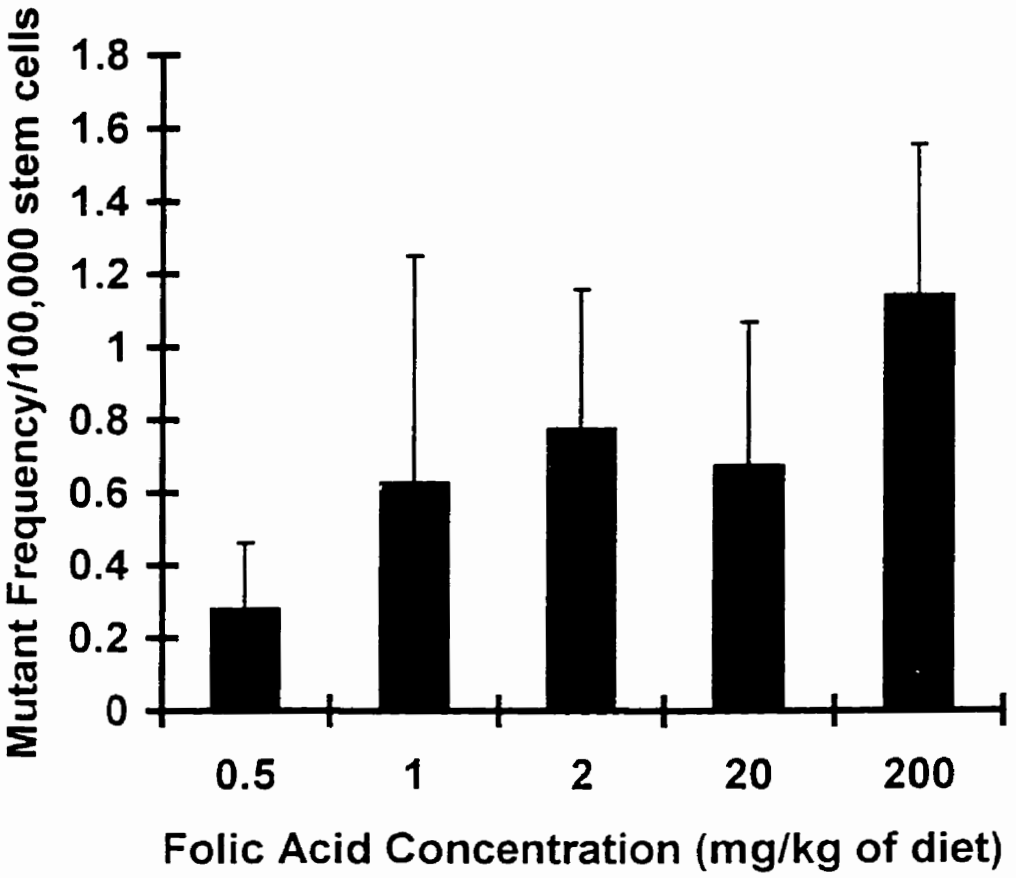
Mutant Frequency/100,000 stem cells
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.5
1
2
20
200
Folic Acid Concentration (mg/kg of diet)

FIGURE 5

CHAPTER 3

DIETARY FLAXSEED REDUCES SPONTANEOUS CHROMOSOME DAMAGE.

(diet, cancer, micronuclei, red blood cells, flow cytometry)

by

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ABSTRACT

Although the incidence of colon and breast cancer are strongly correlated with diet, no specific foods or dietary factors have been identified that account for this correlation. Spontaneous genetic damage provides an alternative model to epidemiological studies and animal experiments with chemical carcinogens for examining dietary correlations. Using flow cytometry to measure micronuclei in peripheral blood erythrocytes, we have found that dietary supplementation of flaxseed can reduce spontaneous chromosome damage in mice. Identification of dietary foods and factors effective at spontaneous reducing genetic damage is a practical approach to cancer prevention.

Epidemiological studies have established environmental factors as important causes of many human cancers and have implicated diet as a major risk factor (1). Diets high in animal fat and low in fiber, fruits, and vegetables are associated with increased risk of breast and colon cancer. Although fat has been thought to be the significant factor, recent studies (2) have cast strong doubt on the specific role of dietary fat, suggesting that an alternate explanation for the epidemiological results is necessary. Diets high in fat may lack adequate amounts of vegetables, fruits, and grains and thus may be deficient in protective factors present in such foods.

Due to the complexity of the human diet and the long interval over which cancer develops, the identification of specific dietary components that are protective can not be determined with confidence based on epidemiological data. Equally, the rarity of spontaneous colon and mammary cancers in laboratory animals has made it difficult to study the cause and effect relationships suggested by human studies. In consequence, most nutritional studies have been conducted on animals treated with high doses of potent initiating carcinogens. While this model may be appropriate for the study of factors involved in cancer promotion, it seems to be a poor model for chronic exposures to initiating carcinogens and inadequate for spontaneous damage. Dietary factors that could reduce such chronic damage substantially may be completely overwhelmed by large doses of a carcinogen. The measurement of genetic damage provides an alternative approach.

Somatic mutations and chromosomal aberrations have a critical role in carcinogenesis (3). There are many such genetic events in a single animal, which makes it possible in principle, to investigate spontaneous events. Nevertheless, these events occur at low frequencies and thus have been difficult to quantify accurately. Micronuclei for example, are found to occur spontaneously in 0.1% to 0.6% of red blood cells of mice (4). These micronuclei originate in the bone marrow when fragments of broken chromosomes or whole chromosomes are left in the cytoplasm at the end of mitosis (5). Measurement of micronuclei in erythrocytes of mice has become one of the standard and most successful methods in genetic toxicology to assay genetic damage *in vivo*. Typically one or two thousand cells are analyzed from each mouse which means only a few spontaneous micronuclei are detected. Recent innovations in the automated measurement of micronuclei in peripheral blood by flow cytometry make it now possible to measure 100 times as many cells which increases the statistical power for the measurements of spontaneous frequencies more that ten fold (6).

Using the peripheral blood micronucleus assay, we have screened 26 foods as nutritional supplements to the standard AIN-93G diet for their effects on spontaneous cytogenetic damage (7). Micronucleus frequencies were measured over a 44-day period in both newly formed cells, the reticulocytes (RET), and the mature or normochromatic erythrocytes (NCE) (6,8). In the mouse, red blood cells are formed in the bone marrow and reach the peripheral circulation in about two days. Reticulocytes can be distinguished from the older erythrocytes based on their high RNA content or high

transferrin receptor content, which is the criterion used in this study (6). As the cell matures, the receptor is lost from the cell surface and the mature erythrocyte remains in circulation for approximately 30 days. Thus a dietary factor that immediately prevented all chromosome damage would eliminate micronuclei in the reticulocytes about three days later and would gradually reduce the frequency in the NCE over the next month. A total of 10,000 reticulocytes and 1,000,000 red blood cells were analyzed per mouse to determine micronucleus frequencies.

The distribution of micronucleus (MN) frequencies in the initial samples of reticulocytes (Figure 1A) approximates the Poisson distribution expected if the only source of variation is random sampling, but some excess variation was detected. The micronucleus frequencies in reticulocytes also fit a normal distribution (n=119; mean=20.3; σ^2=0.002; Kolmogorov-Smirnov, P=0.22). The frequencies of NCE at the start of the experiment were found to significantly deviate from the normal distribution (Figure 1B; n=119; mean=1431.1; σ^2=0.0002; Kolmogorov-Smirnov, P<0.001) with some outliers at higher frequencies and a variance approximately 10 times that expected from sampling alone. Thus most of the variation detected is biological or experimental. It can be seen that initial frequencies fall in a narrow range such that reduction in micronucleus frequency could be detected in principle.

Mice maintained on laboratory chow showed no significant change in micronucleus frequency in either group of cells over the 44 days of the experiment (Figure 2A; Wilcoxon, P=0.14 for RETs; P=0.06 for NCE). Those mice assigned to the

AIN-93G control diet showed a small decline in micronucleated NCE frequencies in the initial study, not observed in a subsequent experiment (Figure 2B). When both sets of data are combined for the control diet, there is an 11% decline in the NCE frequency which is statistically significant (Wilcoxon, P=0.04). The response over time in reticulocytes and red blood cells of mice on AIN-93G was however not significantly different from that observed for the lab chow group (Mann-Whitney, P>0.1 for RET and NCE).

Of the 26 foods tested, flaxseed and rye showed a significant reduction in reticulocyte micronucleus frequency (Wilcoxon, P=0.04 for flaxseed; P=0.04 for rye), not detected in the NCE. In contrast, significant decreases in micronuclei in NCE but not reticulocytes were observed for broccoli (Wilcoxon, P=0.04), soybeans (Wilcoxon, P=0.04), and zucchini (Wilcoxon, P=0.04) supplements. An increase was observed in the micronuclei present in the NCE of mice given cracked wheat supplements (Wilcoxon, P=0.04). Our original expectation had been that a decline or increase would be detected initially in the reticulocytes with a response in the NCE following later in time. After the 44 day period, a similar change in micronucleus frequency was predicted in both cell types. Since this was not observed, a number of foods, including flaxseed, were retested to determine reproducibility of the initial data (9). Results of the initial study and the repetitions are shown in Figure 3. The changes in micronucleus frequencies over time in the second experiment were found not to differ significantly from those of the original study (Mann-Whitney, P>0.06 for all foods). When the two sets of data were combined

for each food, flaxseed was the only food in which a significant decline was detected in both micronucleated NCE (Wilcoxon, P=0.02) and micronucleated reticulocyte (Wilcoxon, P=0.01) frequencies. The combined data show an 11% and 30% reduction in micronucleus frequency in the NCE and reticulocytes, respectively.

Regardless of experiment, the frequency of micronuclei in reticulocytes was found to be higher than in the NCE. This has been observed previously (10) and seems to be characteristic of the mouse strain (C57Bl/6) used. In humans, rats and some species of mice, the spleen rapidly filters out micronuclei from the peripheral circulation so that they are frequent only in the bone marrow. To determine if the difference in micronucleus frequency observed between reticulocytes and NCE was the result of selective removal by the spleen and to confirm previous flaxseed results, micronucleus frequencies were measured over a 44 day period in splenectomized, sham-splenectomized, and control mice (11). Results (Figure 4) show that neither splenectomy nor sham-splenectomy had any effect on the relative frequency of micronuclei in reticulocytes and NCE. Red blood cell biology is evidently influenced in this strain of mice by other unknown biological factors. A decline in micronucleus frequency was observed in the reticulocytes of the sham mice and the NCE of all three groups (Wilcoxon, P=0.002 for sham reticulocytes; Wilcoxon P<0.01 for NCE). When the data from control, sham, and splenectomized mice were pooled, the reduction in micronuclei was significant in the reticulocytes (Figure 5; 18%; Wilcoxon, P=0.0023) and NCE

(16%; Wilcoxon, $P<0.0001$). These results did differ from the AIN-93G control data (Mann-Whitney, $P=0.03$ for both reticulocytes and NCE).

It would be expected that after 44 days comparable responses would be observed in both the reticulocytes and NCE. Furthermore, micronucleus frequencies should stabilize, as cell turnover would have eliminated cells present in the peripheral circulation prior to dietary supplementation. For many foods including flaxseed, the frequencies appeared to continue to decline. Dietary effects on genetic damage may be slow and require longer periods of time than that defined by the life span of the cells. Since a larger number of NCE as compared to reticulocytes were analyzed to determine micronucleus frequency, it would not be unexpected to detect a reduction only in the NCE. Moreover, it would not be unreasonable to detect an effect later in time in the reticulocytes.

The mechanism by which flaxseed is protective against chromosomal damage is not known, nor is the important constituent(s). Flaxseed has been shown previously to inhibit both initiation and promotion stages of carcinogenesis. The incidence of chemically-induced mammary tumors can be reduced by flaxseed feeding (12). The protective effect of flaxseed to chemical carcinogenesis has been attributed in part to mammalian lignans derived from its precursor compounds (13). Epidemiological data has also suggested an important role of lignans in cancer prevention. Urinary excretion of mammalian lignans is significantly lower in breast cancer patients and high risk population groups (omnivores) compared to those with a lower risk such as vegetarians

(14). Flaxseed is the richest source of secoisolariciresinol diglycoside (SD) and other mammalian lignan precursors. The antioxidant property (15) of mammalian lignans is maybe an important factor for the reduction in micronucleus frequencies by flaxseed, as oxidative damage is a significant contributor to spontaneous mutagenesis (16). Lignans, like other phenolic compounds including vitamin E, act as free radical scavengers and inhibit DNA oxidation (17). *In vitro*, phenolic compounds exhibit antimutagenic activity and *in vivo*, they inhibit carcinogen-induced tumors in various tissues including skin, lung and colon. Since flaxseed is composed of various phenolic compounds, in addition to fiber and ω-3 fatty acids, it remains to be determined which constituents contribute to the observed effects.

Micronuclei arise from chromosome breakage and whole chromosome loss events. The use of automated methods for quantifying micronucleated cells is not limited to replacing manual scoring. The size or fluorescence intensity of micronuclei, which are related to its DNA content, can also be measured and provide information as to the mechanism (clastogenic or aneugenic) by which the micronuclei were induced (18). Preliminary data have indicated a small reduction in large size micronuclei in the erythrocytes of mice maintained on flaxseed diets suggesting a protective effect of flaxseed against chromosome loss events.

Our results demonstrate that diet can reduce *in vivo* rates of spontaneous chromosome damage in mice and that a high consumption of flaxseed in particular, can reduce the incidence of micronuclei. The reduction demonstrates an anti-mutagenic role

of diet and lays groundwork in identifying specific dietary factors that are responsible for inhibiting genetic damage. The automated peripheral blood micronucleus assay provides an efficient and alternative method for carrying out nutrition studies. Further studies are required to establish a protective effect of flaxseed in humans. The same endpoint can be measured in splenectomized individuals, so the direct test in humans can be made relatively easily.

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(7) The experiments described here were approved in advance by an independent animal care committee. Care of the animals during the duration of the study was in accordance with committee guidelines. Female C57Bl/6 mice (8 to 9 weeks old; Charles River, St. Constant, PQ) were all housed in standard shoe-box style plastic cages with wood chip bedding at 70 % humidity and a temperature of 22±2 °C with a 12 h light/dark cycle. Mice were randomly assigned to rodent chow (PMI Feeds

Inc., Laboratory Rodent Diet No. 5001), AIN-93G control diet (ICN Biomedicals, Aurora, OH) or one of 26 modified AIN-93G diets containing 20 % of food supplement. Each dietary group contained 5 mice. Freeze-dried fruits and vegetables were purchased from Food Ingredients (Mississauga, ON) and Freeze-Dried Foods (Oakville, ON). All grains except wheat bran and flaxseed were purchased locally. Flaxseed was obtained from Omega Products (Melford, SK) and wheat bran from King Milling (Grand Rapids, MI). Diets were prepared by adding 250 g of powdered test food to 1 kg of powdered AIN-93G diet and kept frozen until use. Water and diet were given *ad libitum* for 30 days with an initial 2-week acclimatization period. Fresh diet was provided every two days. Food consumption for all groups was measured every other day and body weights measured weekly.

(8) Peripheral blood samples were obtained from each mouse on Days 0, 14, 28 and 44 of the study as described (6). Approximately 200 µl of blood was collected from the tail vein into 500 µl of heparin (500 USP Units per ml 0.9 % saline; Orgnanon Teknika, Toronto, ON). An 180 µl aliquot of blood/heparin suspension was then fixed no more that 4 hours from the time of collection in 2 ml of -70 °C absolute methanol. All tubes were stored at -70 °C until they were shipped on dry ice to Litron Laboratories (Rochester, New York) for analysis. To prepare the cells for analysis, 8 ml of ice cold bicarbonate buffer (0.9 % NaCl, 5.3 mM $NaHCO_3$) was added to each tube and the tubes then centrifuged. To differentially stain erythrocyte cell populations, 20 µl aliquots of each cell pellet were added to tubes containing

80 µl fluorescent anti-transferrin receptor antibody (FITC-ATR-Ab; Sigma Chemical, St. Louis, MO) solution with 1 mg RNase A (Sigma Chemical) per ml bicarbonate buffered saline. After 30 minutes at 4 °C, 1 ml cold propidium iodide solution (PI; Sigma Chemical) was added to label micronuclei. Stained cells were then quantitatively analyzed by flow cytometry (FacStar Plus, Becton Dickenson) at 488 nm excitation. A total of 10,000 reticulocytes and 1,000,000 NCE were analyzed to determine micronucleus frequencies.

(9) Follow-up experiments were carried out as previously described (7,8) for broccoli, peas, oranges, wheat bran, flaxseed and AIN-93G control foods. Each group except the control diet contained 5 mice. There were 10 mice in the AIN-93G group.

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(11) Splenectomized, sham and normal female C57Bl/6 mice (8 to 9 weeks old; Charles River, St. Constant, PQ) were all housed as previously described (7). Surgeries were performed in facilities at Charles River. The splenectomized and sham groups each contained 13 mice, while the normal group contained 10. Mice were fed AIN-93G control diet (ICN Biomedicals, Aurora, OH) supplemented 20 % with flaxseed (Omega Products; Melford, SK). Diets were prepared as previously described (7). Water and diet were given *ad libitum* for 30 days with an initial 2-week acclimatization period. Fresh diet was provided every two days. Feed consumption was measured every other day and body weights measured weekly.

Peripheral blood samples were obtained from each mouse on Days 0 and 44 of the study and micronucleus measurements were made as described (6,8).

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FIGURE LEGENDS

Figure 1. Distribution curves of micronucleus frequencies in the Day 0 blood samples measured by flow cytometric methods. (**A**) Frequency distribution for the reticulocyte cell population (n = 119 samples). The expected Poisson distribution is indicated by the curve. (**B**) Frequency distribution for NCE population (n = 119 samples). MN, micronuclei; NCE, normochromatic erythrocytes

Figure 2. Micronucleus frequencies as a function of days on the diet. Change in micronucleated reticulocyte (---) and micronucleated NCE (—) frequencies are shown for (**A**) mice maintained on laboratory rodent chow and (**B**) mice maintained on non-supplemented AIN-93G control diet. Values are means ± SEM (n = five mice for lab chow; five and ten mice for AIN-93G in initial and repeat studies, respectively). Closed diamonds (◆) and triangles (▲) indicate reticulocyte and NCE data obtained in the initial study, respectively. Open squares (□) and circles (○) indicate reticulocyte and NCE data obtained in the repeat study.

Figure 3. Micronucleus frequencies measured by flow cytometric methods for several foods analyzed. Change in micronucleated reticulocyte (---) and micronucleated NCE (—) frequencies over the 44 day period are shown. Values are means ± SEM (n = five mice for all groups except AIN-93G; five and ten mice for AIN-93G in initial and repeat studies, respectively). Closed diamonds (◆) and triangles (▲) indicate reticulocyte and NCE data obtained in the initial study, respectively. Open squares (□) and circles (○) indicate reticulocyte and NCE data obtained in the repeat study.

Figure 4. Relative frequencies of micronuclei in erythrocytes of control, sham and splenectomized C57Bl/6 mice. Reticulocyte (solid bars) and NCE (hatched bars) frequencies are shown for (**A**) Day 0 and (**B**) Day 44. Values are means ± SEM (n = 10 mice for control; 13 mice for sham and splenectomized groups). Splen, splenectomized.

Figure 5. Change in micronucleus frequency of mice maintained on flaxseed supplemented diets. Micronucleated reticulocyte (---) and micronucleated NCE (—) frequencies shown are means ± SEM of pooled data from control, sham, and splenectomized groups (n = 10 mice for control; 13 mice for sham and splenectomized groups). Splen, splenectomized.

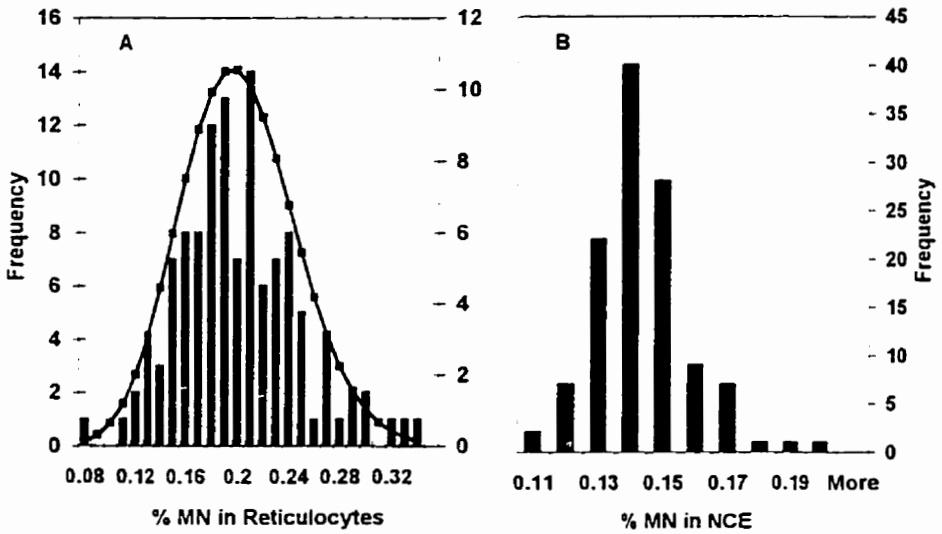

A
B
Frequency
Frequency
16
14
12
10
8
6
4
2
0
12
10
8
6
4
2
0
45
40
35
30
25
20
15
10
5
0
0.08 0.12 0.16 0.2 0.24 0.28 0.32
% MN in Reticulocytes
0.11 0.13 0.15 0.17 0.19 More
% MN in NCE

FIGURE 1

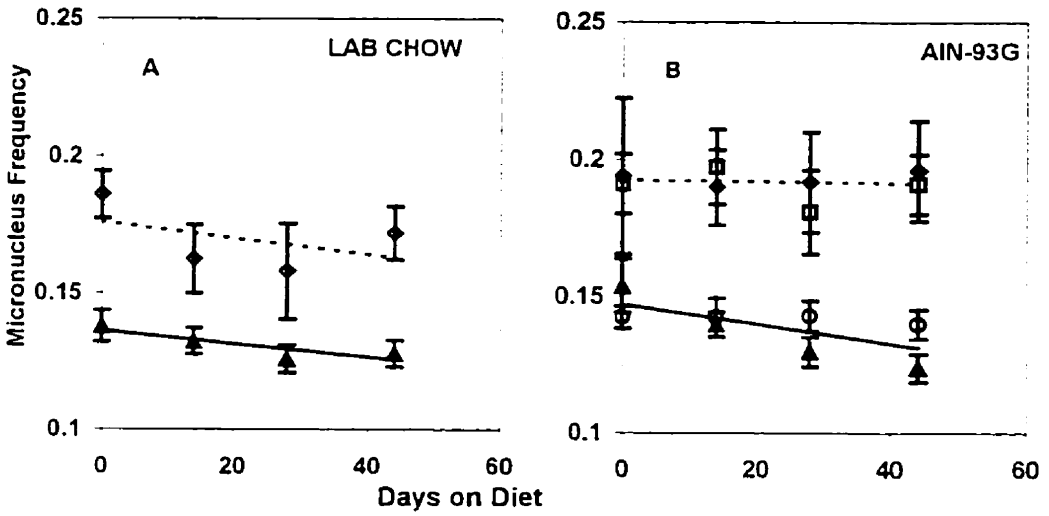
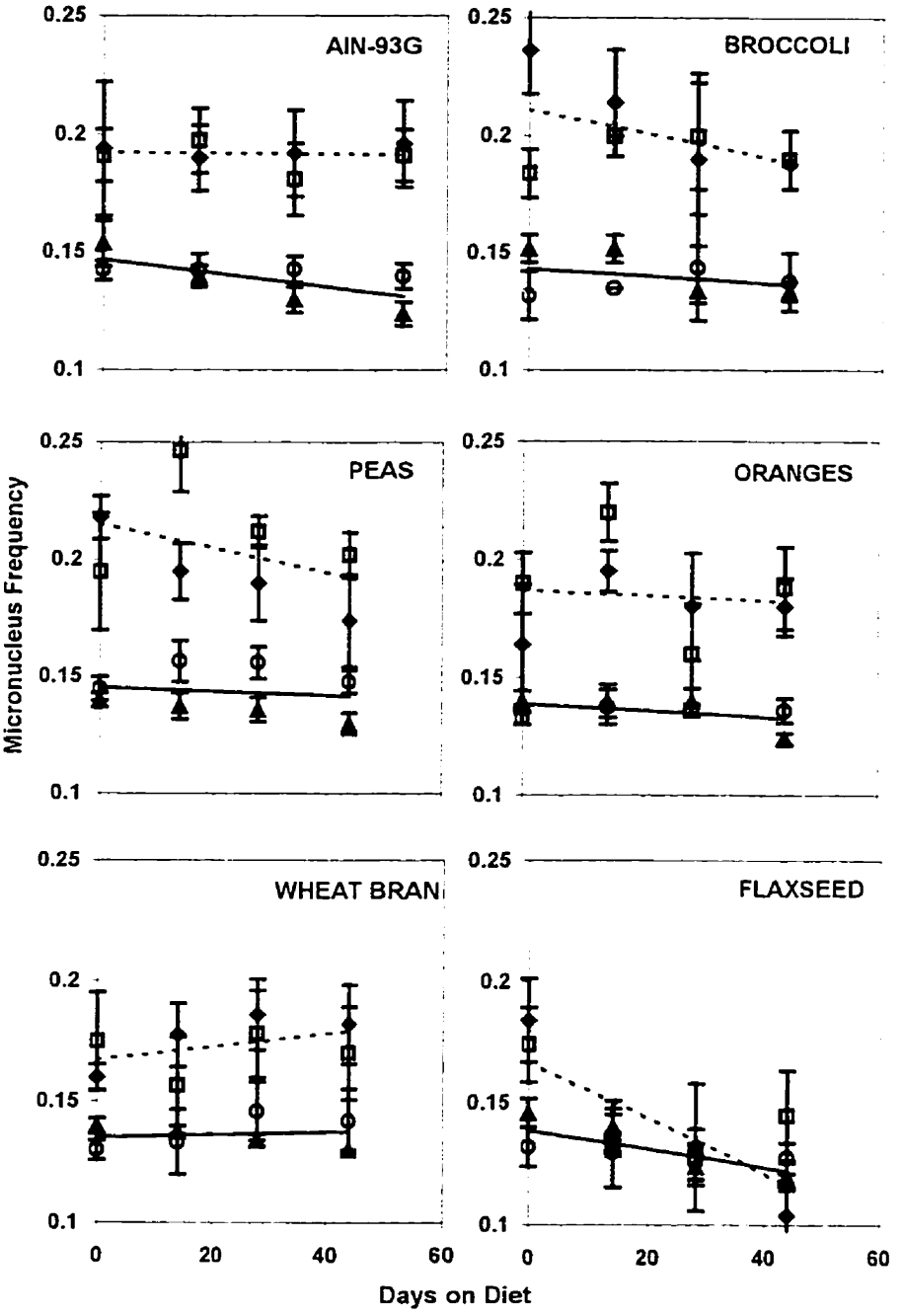

0.25
LAB CHOW
A
Micronucleus Frequency
0.2
0.15
0.1
0
20
40
60
Days on Diet
0.25
AIN-93G
B
0.2
0.15
0.1
0
20
40
60

FIGURE 2


AIN-93G
BROCCOLI
PEAS
ORANGES
WHEAT BRAN
FLAXSEED
Micronucleus Frequency
Days on Diet
0.25
0.2
0.15
0.1
0
20
40
60

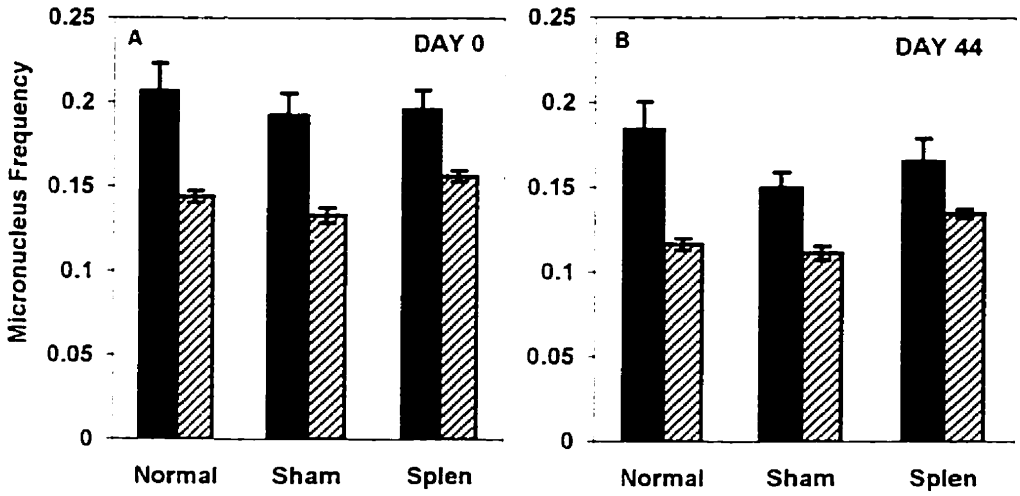

Micronucleus Frequency
0.25
0.2
0.15
0.1
0.05
0
A
DAY 0
Normal
Sham
Splen
B
DAY 44
Normal
Sham
Splen

FIGURE 4

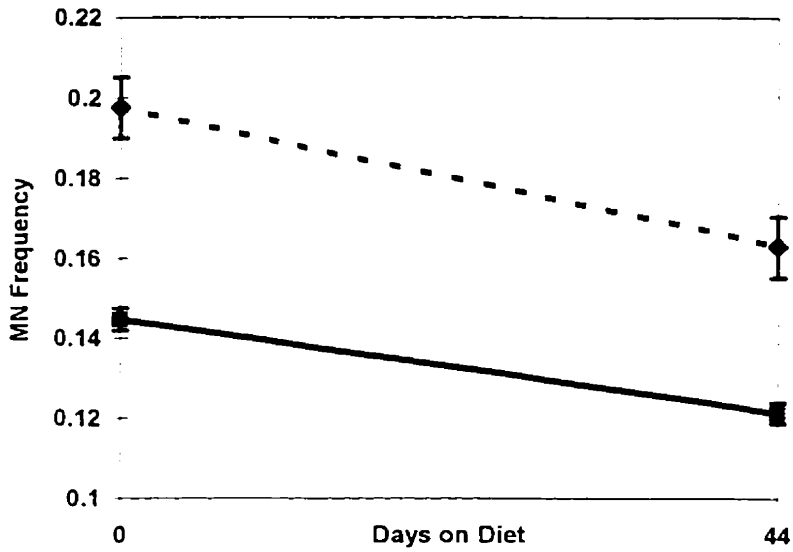


FIGURE 5

CHAPTER 4

EFFECT OF DIETARY SUPPLEMENTATION ON THE FREQUENCY OF SPONTANEOUS *LacZ* MUTATIONS IN THE DEVELOPING COLON

(diet, development, mutation, *lacZ*, colon)

by

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4.1 INTRODUCTION

DNA mutations have a central role in carcinogenesis. Cancer arises from not one but several mutations and in general, those that occur during an individual's lifetime rather than those that are inherited are responsible (Vogelstein et al., 1989; Vogelstein and Kinzler, 1993). The sources of these somatic mutations are replication errors and DNA lesions generated by exogenous and/or endogenous genotoxic agents. Spontaneous damage produced by endogenous factors such as oxygen radicals are particularly important, as they are major contributors of mutations involved in spontaneous carcinogenesis (Ames and Gold, 1990). Although cancers are more common at older ages, most spontaneous mutations arise before adulthood. Two-thirds of the total mutant accumulation at the *hprt* gene in humans occurred by 20 years of age (Finette et al., 1994; Robinson et al., 1994). Further support has come from two transgenic mouse systems. The majority of mutations in these mice arose by 4 week of age with little accumulation later on (Lee et al., 1994; Ono et al., 1995; Paashius-Lew and Heddle, 1996). The events that underlie early development may therefore be significant in determining cancer risk.

The period between conception and birth is characterized by rapid cell proliferation that is unlike any other time in life. This is apparent by the rapid increase in weight during this period (Crispen, 1975). Proliferation is an important factor in both mutagenesis and carcinogenesis (Ames, Gold and Willett, 1995). Increased cell division increases the likelihood of replication errors and the sensitivity of the dividing cells to endogenous and exogenous mutagens while reducing the opportunity for DNA repair

(Ames and Gold, 1991). Therefore the significant rate of mutant accumulation during early life is likely due to effects of increased cell proliferation (Zhang et al., 1995; Heddle et al., 1996; Paashius-Lew and Heddle, 1997).

It is becoming increasingly clear that dietary constituents can inhibit carcinogenesis. Higher vegetable, fruit and/or fiber consumption has consistently been correlated with lower risk of many cancers in both epidemiological studies as well as experimental animal models (Steinmetz and Potter, 1991; Block, Patterson, and Subar, 1992; Wargovich, 1997). The cancer mortality rates of Seventh Day Adventists (SDAs), whom are primarily ovo-lacto-vegetarians, is approximately 50 % of that of the general population (Hocman, 1989). Animal experiments using chemical carcinogens have shown that both the initiation and promotion stages of cancer development can be influenced by dietary factors. Furthermore, numerous *in vitro* and *in vivo* studies with mutagens have demonstrated anti-mutagenic activity of some vitamins and various other dietary constituents of fruits and vegetables (Hayatsu et al., 1988; Odin, 1997). More recently, flaxseed supplemented diets were found to reduce the frequency of spontaneous chromosome damage in erythrocytes of mice (Trentin et al., 1997).

Previous studies of nutrition and cancer have focused primarily on adult rodents and humans. Since most spontaneous mutations arise early in life however, this is the time when dietary anti-mutagens and anti-carcinogens may be most needed and are most effective. By reducing somatic mutation rates during development, nutritional factors can possibly reduce the risk of cancer in later life. To examine effect of dietary

supplementation on spontaneous mutation frequencies during development, mutant frequencies were measured in 3 and 8 week-old mice whose mothers were fed high vegetable, fruit or flaxseed diets during pregnancy and lactation. Since diet is a major factor in colon cancer, mutation frequencies were measured at the *lacZ* transgene in the epithelial cells of mouse colon using the Muta™ Mouse transgenic system.

4.2 MATERIALS AND METHODS

Animals and Diets. The experiments reported here were approved in advance by the animal care committee of York University and conformed to the Canadian guidelines for animal care.

All mice used in the experiments were the F_1 offspring of SWR females (Jackson Laboratories, Bar Harbor ME) and Muta™ Mouse males (Hazelton Research, VA). All F_1 offspring were hemizygous for the *lacZ* transgene. Components of the AIN-93G rodent diet were purchased from ICN Biomedicals (Aurora, OH) and freeze-dried fruits and vegetables were obtained from either Food Ingredients (Mississauga, ON) or Freeze-Dried Foods (Oakville, ON). Flaxseed was purchased from Omega Products (Melford, SK).

The mice were housed in standard shoe-box style plastic cages with wood chip bedding, at 70 % humidity and a temperature of 22±2 °C with a 12 h light/dark cycle. Diet and water were fed *ad libitum*. The SWR females were randomly assigned to either the AIN-93G control diet or one of 9 modified AIN-93G diets containing 25% (w/w) of food agent. Chemical composition was determined for each food agent and proteins, carbohydrates and fats were adjusted to make all diets isocaloric. Prepared diets were stored at -20 °C until use. Feed consumption was measured every other day and body weights were measured weekly.

Three SWR females were bred with one Muta™ Mouse male for each treatment group. The male mouse was removed once a visible sign of pregnancy was observed and pregnant females were then housed individually. The females continued on the assigned diets during the entire duration of pregnancy and lactation. Half of the offspring of each dam were sacrificed at 3 weeks of age for mutation analysis and the remainder continued on the diets assigned until 8 weeks of age, at which time they were sacrificed. The number and gender of the pups analyzed at 3 and 8 weeks depended upon the gender composition and litter size in each treatment group.

The *LacZ* Transgenic Mouse Assay. Genomic DNA was extracted from colonic epithelial cell suspensions. After sacrifice, the entire colon was removed, flushed with phosphate buffered saline (PBS; pH 8.0) and inverted. The tissue was then placed in 2 ml

of 75 mM KCl/20 mM EDTA solution and gently forced in and out of a 5 cc needless syringe to remove the epithelial cells from the colon.

Genomic DNA was extracted by proteinase K (2 mg/ml, Sigma) cell digestion, phenol-chloroform extraction and ethanol precipitation as described previously by Trentin and Heddle (1997). The lambda phage shuttle vector, which contains the entire the entire *lacZ* mutational target gene, was recovered from mouse DNA by *in vitro* packaging with Transpack® Packaging Extract (Stratagene, La Jolla, CA). *LacZ* mutations were detected by incubating the extracts with *E.coli* C (amp^r, kan^r, lac-, galE-, recA-) and plating the infected bacteria on LB plates containing 0.003 % phenyl β-*D*-galactopyranoside (p-gal; Sigma, St. Louis, MO) as a selection agent. Mutated *lacZ* genes produce no functional β-*D*-galactosidase. If lambda phage with a mutant *lacZ* gene is then plated in the presence of a lactose analog such as p-gal, a plaque forms. If the *lacZ* gene is functional however, plaque formation is prevented because cleavage of the β-*D*-galactoside bond results in a toxic accumulation of UDP-galactose due to a *galE* mutation in the *E.coli*. Thus, only mutants can develop plaques in the presence of p-gal. To determine the titre for the phage reaction mixture and thus determine the number of loci analyzed, infected *E.coli* was also plated under non-selective conditions.

Statistics Results were analyzed by t-tests and one-way analysis of variance using the statistical program in Microsoft Excel Software. All means and standard errors reported

are based on the mutant frequency observed in each animal, regardless of the number of plaques analyzed.

4.3 RESULTS

All mutation data collected for 3 and 8 week-old offspring using the Muta™ Mouse transgenic system are shown in Tables 14 to 15 of Appendix A. Litter sizes were within normal ranges for all dams and no significant differences in weight gain of dams or pups were observed between groups. Mutant frequencies of pups from one mother were found not to be statistically significant from pups of another mother in the same treatment group for all data analyzed. The consistency in mutant frequencies between pups of the same group supports a primary role of the maternal diet on the observed frequencies.

Spontaneous *lacZ* mutant frequencies in 3 week-old colon are shown in Figure 1. Mutant frequencies are significantly elevated in mice fed broccoli (F_B=8.83; DF=1, 23; P=0.007) and oranges (F_O=7.45; DF=1, 23; P=0.011) supplemented diets as compared to mice in the control group. A higher mutant frequency is characteristic of all pups within the broccoli group indicating the lack of any protective effect by this food. Only two (2) of the five (5) pups maintained on oranges had mutant frequencies that were elevated. Since the two pups were not siblings, the low plaque titres obtained for each of these mice likely contributed to a greater variance in mutant frequencies. A more accurate measure of spontaneous mutant frequency in these pups would require a greater number

of *lacZ* transgenes to be analyzed. Mutant frequencies of mice in the other dietary groups were no different from the mutant frequency observed in the control mice (F=1.41; DF=7, 52; P=0.22).

Spontaneous mutant frequencies for 8 week-old offspring are shown in Figure 2. Mutant frequencies of mice fed the green pepper supplemented diet were significantly lower compared to those of control mice (F=13.05; DF=1, 12; P=0.004). In contrast, mutant frequencies of mice maintained on control, control with 9 % fat or flaxseed diets did not differ from each other (F=2.65; DF=2, 16; P=0.10). When 3 week data were compared to its 8 week counterpart, the mutant frequencies between the two ages were significantly different for control with 9 % fat (F=13.5; DF=1, 9; P=0.005) and flaxseed mice (F=9.41; DF=1, 12; P=0.01). The control frequency at 8 weeks is higher than that observed at 3 weeks but was found not to be significantly different. The 8 week mutant frequency is a reflection of the number of mutations that have accumulated to this time. Since mutations accumulate with age, a higher frequency at 8 weeks such as that observed with flaxseed and control with 9 % fat is expected. With respect to the green pepper, two observations suggest the results are not real effects and are attributed to statistical factors. First, the mutant frequency at 8 weeks of age is lower than that at 3 weeks. Second, the 8 week frequency is lower than the control frequency, an effect not seen in the younger mice.

4.4 DISCUSSION

The identification of dietary inhibitors of mutagenesis is of significant importance for cancer prevention. Transgenic mice were used to examine the effects of dietary supplementation during pregnancy and lactation on the frequency of spontaneous somatic mutations in offspring. Previous studies in our laboratory examining the accumulation of mutations during development in *lacZ* mice, have shown that the mutant frequency of 3 week-old pups to be significantly higher than control frequencies observed here (Figure 4; Paashuis-Lew and Heddle, 1997). Mice in that study were fed laboratory rodent chow (Purina 5001) while in the present study the basal diet was AIN-93G (ICN Biomedicals), specially formulated for growth, pregnancy and lactation. Concentrations of dietary factors such as folic acid, vitamin E and linolenic acid in this diet are much higher in order to accommodate increased fetal demands and ensure normal growth. The anti-mutagenic activity of many of these dietary constituents have been shown in numerous *in vitro* and *in vivo* studies with mutagens (Hayatsu et al., 1988; Odin, 1997).

Neither flaxseed nor any of the vegetables or fruits tested in this study were found to reduce the mutant frequency in the colon below non-supplemented levels. The lack of any protective effect by these foods indicates that the composition of AIN-93G diet may alone be adequate to limit spontaneous DNA damage to a level that the remaining mutations that arise are due only to lesions that can not be prevented by nutritional factors such as replication errors. Therefore, any additional supplementation of dietary anti-mutagens to the control diet is of no greater benefit with respect to decreasing

mutation rates. The minimum dietary concentration of nutrients supplied by the laboratory rodent chow is sufficient to prevent any nutritional diseases in rodents but is insufficient to reduce the spontaneous mutation frequency.

These findings can be extended to explain diet and cancer correlations seen in the human population. The Western-style diet for the most part, provides the recommended daily requirement of nutrients to prevent deficiency diseases such as scurvy and rickets but is inadequate to reduce genetic damage in humans. In contrast, the high vegetable and fruit diet of SDAs provides concentrations of vitamins and other dietary factors well above the minimum allowances and as a result, mutation rates are likely to be lower contributing to significantly lower cancer rates. Lower sister-chromatid exchange frequencies have been demonstrated in SDAs as compared to the general population supporting such a hypothesis (Wulf et al., 1986).

The results of this study have shown that maternal diet can be an important factor in the accumulation of mutations in the offspring. Furthermore, these results have suggested that deficiencies in the diet can increase the incidence of somatic mutations and consequently, may give rise to increase cancer rate. Additional studies with transgenic mouse systems examining the effects of specific nutrient deficiencies on mutation frequencies, would be important in identifying those dietary factors whose increased consumption reduces mutation rates and thus, cancer risk.

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FIGURE LEGENDS

Figure 1. Spontaneous *lacZ* mutant frequencies in the colon of 3 week-old mice fed AIN-93G control or supplemented diets during development. Values are means ± SEM.

Figure 2. Spontaneous *lacZ* mutant frequencies in the colon of 8 week-old mice fed AIN-93G control or supplemented diets during development. Values are means ± SEM of available data.

Figure 3. Comparison of *lacZ* mutant frequencies of 3 week-old mice fed laboratory rodent chow and those fed AIN-93G control or supplemented diets during development. Mutant frequency for lab chow is the mean (± SEM) of original data from Paashuis-Lew and Heddle (1997) and data obtained upon repackaging of the DNA. For all other groups, values are the means ± SEM. Broccoli and orange mutant frequencies are not shown as the observed frequencies are not attributed to diet.

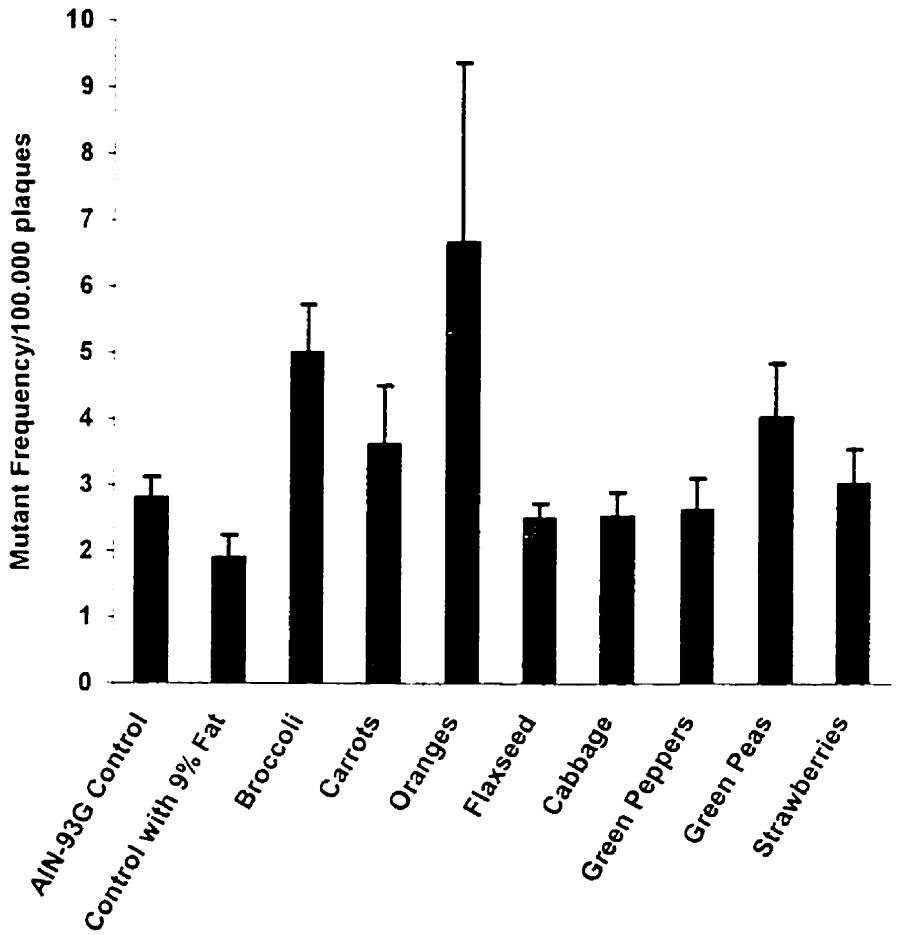
Mutant Frequency/100,000 plaques
10
9
8
7
6
5
4
3
2
1
0
AIN-93G Control
Control with 9% Fat
Broccoli
Carrots
Oranges
Flaxseed
Cabbage
Green Peppers
Green Peas
Strawberries

FIGURE 1

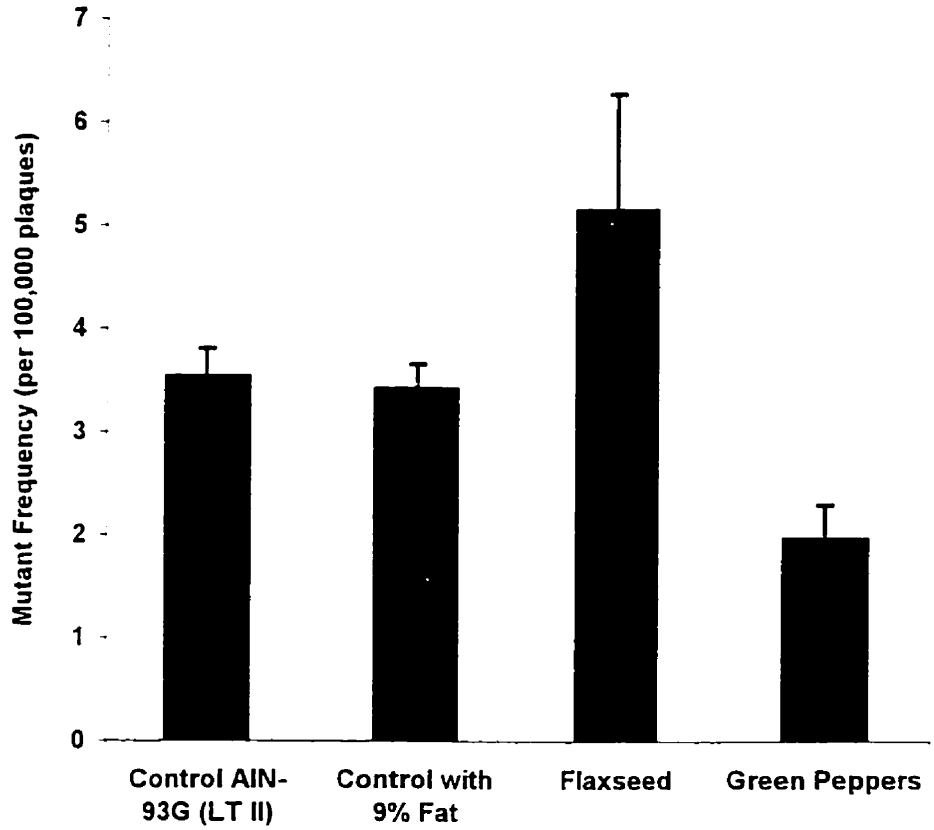
Mutant Frequency (per 100,000 plaques)
7
6
5
4
3
2
1
0
Control AIN-93G (LT II)
Control with 9% Fat
Flaxseed
Green Peppers

FIGURE 2

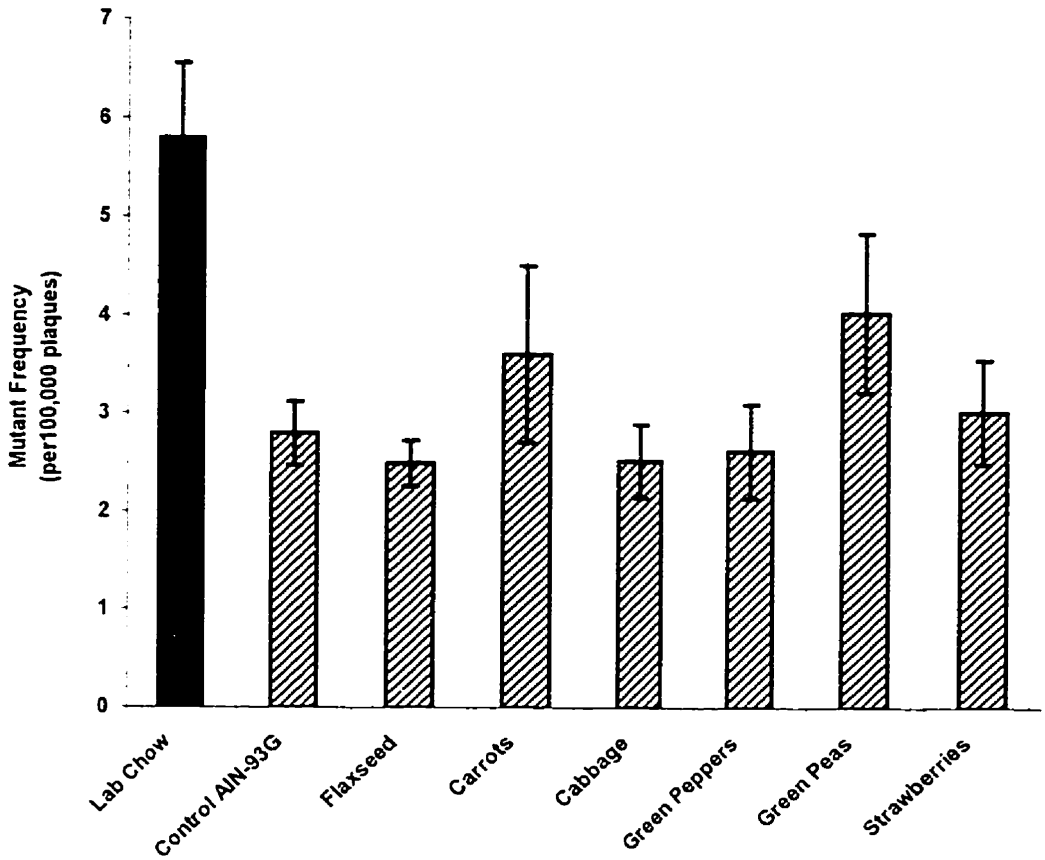
Mutant Frequency
(per100,000 plaques)
7
6
5
4
3
2
1
0
Lab Chow
Control AIN-93G
Flaxseed
Carrots
Cabbage
Green Peppers
Green Peas
Strawberries

FIGURE 3

CHAPTER 5

SUMMARY AND GENERAL DISCUSSION

Population studies have demonstrated that dietary modifications can reduce the incidence of many cancers. The human diet however, is a complex mixture of nutrients and identifying with confidence the specific dietary factors that are protective, is difficult. Animal models have been useful to study the associations suggested by epidemiological data since diets can be adjusted to test the effects of specific constituents. However, the high doses of carcinogens used in these experiments limit the extent to which the data can be extrapolated to humans.

Somatic mutations have a central role in cancer development, suggesting the use of *in vivo* mutation assays as an alternative approach to studying dietary correlations. Spontaneous genetics events are rare and thus difficult to quantify accurately. Recent developments in mutational assays however, now make it possible to study dietary effects on mutations in the absence of mutagen treatment. The micronucleus assay is used extensively in genetic toxicology to measure chromosome damage *in vivo*. The introduction of automated methods for quantifying micronuclei in peripheral blood by flow cytometry, have increased the statistical power of detecting reductions in genetic damage as compared to manual scoring (Tometsko et al., 1993; Dertinger et al., 1996; Grawé et al., 1997). The development of transgenic mouse systems to study the accumulation of mutations by various mutagens, can also be extended to study anti-mutagenesis. Mutation frequencies can be measured *in vivo* in any cell or tissue type, under conditions that closely reflect the human situation in terms of metabolism and distribution.

Nutrition studies have focused primarily on adults, although most spontaneous mutations arise during development. Mutant accumulation during this period can be an important factor in determining an individual's risk of cancer in later life. Therefore, early life is the time when dietary anti-mutagens and anti-carcinogens are most needed and should be most effective.

The chromosome damaging effects of low folate have been demonstrated both *in vitro* and *in vivo* (Reidy, et al., 1983; Everson et al., 1988; MacGregor et al., 1990). Folic acid deficiency is the most common vitamin deficiency in the world, especially among pregnant women, yet the effects of low maternal folate levels on chromosome damage and mutations in developing animals have not been examined. Completely deficient diets are detrimental to reproduction because of the essential role of folate in cell metabolism. Therefore, while such conditions can be used on adults, concentrations of folic acid below 0.5 mg/kg are intolerable for development studies. Results from the micronucleus assay and the Muta™ Mouse transgenic system have shown that the frequency of genetic damage and mutation is similar in offspring whose mothers were on low folate diet during pregnancy and lactation as compared to those on high folate. Concentrations of folic acid at levels 400 times higher than the minimum dietary requirement did not prove to be beneficial with respect to reducing genetic damage. The data on effects of folic acid supplements on carcinogenesis have been inconsistent indicating that additional studies are necessary.

The limitation to mouse transgenic systems is the time and expense required in measuring mutations. Since chromosomal aberrations are involved in carcinogenesis, the micronucleus assay was used to screen 26 foods for their effects on spontaneous genetic damage. Automated methods for measuring micronucleus frequencies were incorporated to increase the accuracy and reliability of the results. After 44 days, a significant reduction in both micronucleated reticulocyte and red blood cell frequencies was observed in mice maintained on high flaxseed diets. Flaxseed has been shown previously to inhibit initiation and promotion stages of carcinogenesis (Serraino and Thompson, 1992; Jenab and Thompson, 1996) suggesting both an anti-mutagenic and anti-carcinogenic property for flaxseed. The absence of comparable responses in reticulocytes and NCE for several foods has suggested that dietary effects as compared to those of chemical mutagens, may be slow and require longer time than that defined by our experiments. Many of the frequencies appeared to continue to decline even after 44 days, indicating a steady state had yet to be achieve. Therefore, future dietary studies incorporating the micronucleus assay should extend the time of dietary intervention (> 2 months).

Since flaxseed is effective at reducing spontaneous chromosome damage, it may also be effective at decreasing mutant accumulation during development. The absence of any significant reduction in the mutant frequency of the offspring by dietary supplementation of flaxseed or one of 9 other foods during pregnancy and lactation, suggests these foods have no protective effect. Mutant frequencies were significantly

lower however than those of a previous study (Paashuis-Lew and Heddle, 1997) in which mice were fed laboratory rodent chow. An explanation for the results is that the basal diet in the present study (AIN-93G) is nutritionally better than the regular laboratory chow. The higher concentration of nutrients in the AIN-93G diet is sufficient to reduce the spontaneous frequency of mutations to a level where any additional mutations that are observed are due solely to lesions that diet can not be prevented. Further dietary supplementation is therefore of no greater benefit. Most human diets are not nutritionally sufficient. The reason high fat diets are correlated with cancer risk may be that these diets lack adequate amounts of vegetables and fruits and thus are deficient in protective nutrients in these foods. Similar studies could be directed at examining the effects of nutritional deficiencies on mutation frequencies as a means of identifying those constituents whose increased consumption reduces mutation rates.

These studies have demonstrated that diet can reduce *in vivo* rates of spontaneous genetic damage and consequently, the resulting somatic mutation frequencies. Furthermore, it has been shown that maternal nutrition is an influential factor for the accumulation of mutations during development. Dietary modification by women during pregnancy and lactation may be beneficial to potentially reducing the incidence of cancer in their offspring. Future studies should be directed at establishing a protective effect of flaxseed in humans. A direct test can be made using the micronucleus assay in splenectomized individuals. Furthermore, fractionation of flaxseed is required to isolated the important constituent or constituents responsible for reducing mutagenesis and

possibly carcinogenesis. Since the cooking of some foods produces mutagens such as heterocyclic amines, it would be of interest to examine the effects of fruit and vegetable supplemented diets on the frequency of mutations induced by these mutagens.

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APPENDIX A

ORIGINAL DATA

Table 1. Micronucleus frequencies in the NCE and reticulocytes (PCE) of 3 week-old mice as a function of folic acid concentration.

		MN Frequency per 1000 Cells	
Treatment	**Tag#**	**NCE's**	**PCE's**
A: 0.5 mg/kg of diet	2902	4.15	2.12
	2904	3.62	1.73
	2909	2.46	1.09
	2912	2.44	2.59
	2913	2.35	4.60
	3137	3.47	1.64
B: 1.0 mg/kg of diet	2905	6.25	0.97
	3100	6.42	0.90
C: 2.0 mg/kg of diet	2988	2.78	2.57
	2993	2.19	0.74
	3081	2.94	1.97
D: 20 mg/kg of diet	2995	3.03	3.97
	2999	2.48	2.59
	3090	4.19	3.79
E: 200 mg/kg of diet	2923	2.28	0.00
	2925	2.92	2.69
	2915	2.90	1.52
	2917	2.09	1.60
	2921	3.27	3.47
	3092	2.85	0.99

Table 2. Micronucleus frequencies in the NCE and reticulocytes (PCE) of 8 week-old mice as a function of folic acid concentration.

		MN Frequency per 1000 Cells	
Treatment	**Tag#**	**NCE's**	**PCE's**
A: 0.5 mg/kg of diet	2933	2.07	2.99
	2935	2.87	1.96
	2937	2.13	0.99
	2938	3.80	0.98
	2941	2.67	2.86
	3136	4.25	0.92
	3138	3.46	2.05
B: 1.0 mg/kg of diet	2598	2.32	2.98
	3098	2.69	0.91
	3099	3.09	0.94
C: 2.0 mg/kg of diet	2989	4.07	3.53
	2991	3.43	0.99
	3080	2.59	0.00
D: 20 mg/kg of diet	2998	1.63	1.00
E: 200 mg/kg of diet	2952	1.85	0.00
	2955	2.70	1.00
	2957	2.26	1.23
	2944	2.16	0.00
	2950	2.50	2.00
	3094	2.34	2.00

Table 3. Spontaneous *lacZ* mutation frequencies in the small intestine of 3 week-old mice as a function of folic acid concentration.

Treatment	Tag#	Total # of Plaques	# Mutants	Mutant Frequency (x10-5)
A: 0.5 mg/kg diet	2901	145000	4	2.8
	2902	140000	2	1.4
	2904	105000	1	1.0
	2909	197500	5	2.5
	2912	132500	3	2.3
	2913	117500	2	1.7
	3137	52500	1	1.9
	3139	45000	1	2.2
B: 1.0 mg/kg diet	2905	97500	2	2.1
	3100	30000	0	0.0
C: 2.0 mg/kg diet	2988	195000	6	3.1
	2992	225000	5	2.2
	2993	95000	3	3.2
	3081	162500	12	7.4
	3083	135000	6	4.4
	3086	140000	6	4.3
D: 20 mg/kg diet	2995	65000	1	1.5
	2999	157500	7	4.4
	3089	22500	1	4.4
	3090	147500	1	0.7
E: 200 mg/kg diet	2923	100000	15	15.0
	2924	135000	5	3.7
	2925	152500	4	2.6
	2915	255000	2	0.8
	2917	90000	4	4.4
	2918	112500	7	6.2
	2921	100000	13	13.0
	3095	70000	1	1.4
	3096	122500	4	3.3

Table 4. Spontaneous *lacZ* mutation frequencies in the small intestine of 8 week-old mice as a function of folic acid concentration.

Treatment	Tag#	Total # of Plaques	# Mutants	Mutant Frequency (x10-5)
A: 0.5 mg/kg diet	2933	25000	3	4.0
	2935	75000	0	0.0
	2940	30000	0	0.0
	2941	27500	2	7.3
C: 2.0 mg/kg diet	2989	57500	2	3.5
	2990	37500	2	5.3
	2991	15000	0	0.0
E: 200 mg/kg diet	2952	55000	4	7.3
	2955	47500	0	0.0
	2957	47500	2	4.2
	2944	32500	2	6.2
	2950	65000	3	4.6

Table 5. Spontaneous *lacZ* mutant frequencies in the colon of 3 week-old mice as a function of folic acid concentration.

Treatment	Tag#	Total # of Plaques	# Mutants	Mutant Frequency (x10-5)
A: 0.5 mg/kg diet	2901	82500	2	2.4
	2902	80000	1	1.3
	2904	92500	3	3.2
	2909	35000	0	0.0
	2912	82500	4	4.9
	2913	102500	0	0.0
	3137	75000	0	0.0
	3139	140000	1	0.7
B: 1.0 mg/kg diet	2905	97500	1	1.0
	3100	102500	1	1.0
C: 2.0 mg/kg diet	2988	75000	1	1.3
	2992	82500	3	3.6
	2993	95000	1	1.1
	3081	115000	2	1.7
	3083	95000	0	0.0
	3086	85000	3	3.5
D: 20 mg/kg diet	2995	82500	3	3.6
	2999	107500	1	0.9
	3089	80000	1	1.3
	3090	110000	1	0.9
E: 200 mg/kg diet	2923	145000	1	0.7
	2924	80000	2	2.5
	2925	120000	3	2.5
	2915	100000	3	3.0
	2917	57500	1	1.7
	2918	110000	3	2.7
	2921	135000	3	2.2
	3095	135000	1	0.7
	3096	177500	2	1.1

Table 6. Spontaneous *lacZ* mutation frequencies in the colon of 8 week old mice as a function of folic acid concentration.

Treatment	Tag#	Total # of Plaques	# Mutants	Mutant Frequency (x10-5)
A: 0.5 mg/kg diet	2933	110000	7	6.4
	2935	95000	2	2.1
	2936	90000	3	3.3
	2938	132500	3	2.3
	2940	57500	0	0.0
	3136	95000	2	2.1
	3138	97500	4	4.1
B: 1.0 mg/kg diet	2598	142500	1	0.7
	3098	60000	0	0.0
C: 2.0 mg/kg diet	2989	80000	1	1.3
	2990	27500	0	0.0
	2991	142500	7	4.9
	3080	80000	3	3.8
	3085	107500	2	1.9
	3087	67500	4	5.9
D: 20 mg/kg diet	2996	122500	3	2.4
	2997	80000	4	5.0
	2998	90000	2	2.2
	3088	85000	2	2.4
E: 200 mg/kg diet	2955	50000	1	2.0
	2957	165000	4	2.4
	2944	17500	0	0.0
	2950	117500	4	3.4
	3091	40000	2	5.0
	3093	117500	6	5.1
	3094	147500	0	0.0

Table 7. Spontaneous mutation frequencies at the *Dlb-1* locus in 8 week-old mice as a function of folic acid concentration.

Treatment	Tag#	# Villi Scored	# Mutants	Mutant Frequency (x10-4)
A: 0.5 mg/kg diet	2933	23004	2	0.9
	2935	9181	1	1.1
	2936	14834	0	0.0
	2937	8399	0	0.0
	2940	19631	0	0.0
	2941	12888	0	0.0
	3136	14125	0	0.0
B: 1.0 mg/kg diet	2598	16003	2	1.3
	3098	10481	0	0.0
C: 2.0 mg/kg diet	2989	15782	2	1.3
	2990	11600	0	0.0
	2991	8757	2	2.3
	3080	18437	0	0.0
	3085	6058.5	1	1.1
	3087	14958	0	0.0
D: 20 mg/kg diet	2996	8737	1	1.2
	2997	11925	0	0.0
	2998	13019	2	1.5
	3088	13464	0	0.0
E: 200 mg/kg diet	2952	13545	3	2.2
	2955	10575	0	0.0
	2957	13050	3	2.3
	2944	9141	0	0.0
	3091	9809	1	1.0
	3097	7657	1	1.3

Table 8. Micronucleated reticulocyte frequencies measured over 44 days in mice fed vegetable, fruit or grain supplemented diets.

		MN-RET Frequency (% per 10,000 total reticulocytes)			
Treatment	**Tag #**	**Day 0**	**Day 14**	**Day 28**	**Day 44**
AIN-93G Control	3121	0.21	0.19	0.2	0.17
	3109	0.19	0.17	0.17	0.17
	3126	0.29	0.24	0.25	0.25
	3124	0.16	0.19	0.14	0.16
	3133	0.12	0.16	0.2	0.23
Apples	3170	0.15	0.12	0.19	0.19
	3142		0.18	0.16	0.12
	3157		0.14	0.26	0.15
	3152		0.15	0.15	0.1
	3163	0.24	0.18	0.12	0.1
Asparagus	3183	0.17		0.15	0.2
	3195	0.18	0.27	0.23	0.24
	3200	0.18	0.21	0.16	0.22
	3205	0.2	0.18	0.21	0.17
	3217	0.24	0.21	0.21	0.25
Barley	3187	0.25	0.16	0.12	0.21
	3189	0.17	0.16	0.17	0.21
	3219	0.29	0.15	0.17	0.16
	3204	0.24	0.15	0.12	0.17
	3207	0.24	0.13	0.17	0.19
Beets	3194	0.23	0.26	0.22	0.14
	3196		Tail Sliced		
	3215	0.23	0.15	0.21	0.19
	3208	0.24	0.17	0.15	0.21
	3202	0.21	0.21	0.14	0.25

Table 8. continued

Black Tea	3284	0.18	0.17	0.18	
	3283	0.19	0.16	0.16	0.22
	3275	0.14	0.22	0.23	0.18
	3258	0.17	0.26	0.17	0.24
	3271	0.2	0.28		0.22
Broccoli	3117	0.18	0.19	0.19	0.24
	3122	0.21	0.17	0.24	0.29
	3106	0.25	0.24	0.15	0.19
	3130	0.33	0.27	0.13	0.08
	3131	0.21	0.2	0.24	0.14
Brussel Sprouts	3173	0.28	0.22	0.17	0.18
	3141		0.13	0.19	0.15
	3154		0.15	0.24	0.13
	3150		0.18	0.13	0.21
	3169		0.23	0.14	0.26
Cabbage	3197	0.13	0.22	0.2	0.21
	3188	0.21	0.24	0.11	0.17
	3199	0.22	0.23	0.16	0.14
	3203	0.23	0.2	0.13	0.16
	3209	0.27	0.19	0.15	0.21
Canteloupe	3282	0.27	0.24	0.22	0.22
	3279	0.2	0.19		0.2
	3281	0.19	0.2	0.2	0.2
	3268	0.19	0.2	0.29	0.21
	3264	0.2	0.22	0.22	0.19

Table 8. continued

Cauliflower	3198	0.19	0.2	0.11	0.15
	3182	0.14	0.13	0.14	0.18
	3212	0.2	0.17	0.14	0.17
	3216	0.18	0.15	0.2	0.2
	3220	0.21	0.18	0.12	0.2
Carrots	3112	0.23	0.17	0.18	0.16
	3114	0.19	0.25	0.16	0.16
	3102	0.15	0.18	0.2	0.24
	3128	0.15	0.15	0.14	0.15
	3135	0.16	0.21	0.18	0.13
Corn	3174	0.32	0.2	0.21	0.19
	3140		0.17	0.21	0.21
	3156		0.13	0.17	0.19
	3159		0.21	0.19	0.2
	3167	0.12	0.16	0.24	0.25
Flaxseed	3101	0.13	0.11	0.11	0.1
	3125	0.21	0.13	0.17	0.09
	3129	0.18	0.16	0.06	0.07
	3113	0.23	0.16	0.11	0.12
	3107	0.17	0.13	0.21	0.14
Green Beans	3171	0.25	0.23	0.21	0.23
	3144	0.34	0.2	0.2	0.14
	3151		0.18	0.14	0.18
	3161		0.22	0.23	0.17
	3166	0.15	0.14	0.17	0.21

Table 8. continued

Green Peas	3123	0.21	0.18	0.18	0.13
	3105	0.2	0.22	0.19	0.18
	3103	0.24		0.24	0.13
	3118	0.2	0.17	0.14	0.23
	3111	0.24	0.21	0.2	0.2
Green Peppers	3192	0.11		0.23	0.16
	3185	0.19	0.21	0.11	0.2
	3206	0.3	0.3	0.26	0.21
	3218		Tail Sliced		
	3210	0.25	0.19	0.2	0.23
Green Tea	3289	0.15	0.15	0.22	0.19
	3278	0.19	0.17	0.23	
	3280	0.17	0.24		0.19
	3266	0.24	0.23		
	3263	0.16	0.21	0.15	0.15
Lentils	3143		0.15	0.16	0.15
	3148		0.18	0.2	0.16
	3155		Dead		
	3165	0.16	0.19	0.19	0.19
	3160	0.22	0.15	0.17	0.18
Oats	3290	0.18	0.16	0.16	0.14
	3273	0.19	0.21		0.16
	3274			0.13	0.18
	3267	0.17	0.21		0.19
	3269	0.13	0.11	0.13	0.18

Table 8. continued

Oranges	3108	0.21	0.2	0.19	0.18
	3104	0.13		0.11	0.2
	3115	0.21	0.21	0.21	0.17
	3119	0.08	0.2	0.24	0.14
	3134	0.19	0.17	0.15	0.21
Potato	3149		0.21	0.15	0.13
	3146		0.15	0.19	0.13
	3158	0.22	0.12	0.15	0.21
	3168	0.18	0.19	0.13	0.11
	3164		0.18	0.24	0.16
Rye	3184	0.25	0.16	0.14	0.1
	3191	0.18	0.18	0.14	0.17
	3193	0.21	0.15	0.19	0.15
	3213	0.18	0.09	0.13	0.14
	3214	0.22	0.13	0.18	0.19
Soybeans	3285	0.18	0.17	0.15	0.14
	3286	0.23	0.25	0.18	0.16
	3277	0.22	0.18		0.2
	3272	0.18	0.15	0.22	0.18
	3265	0.19	0.21	0.2	0.25
Spinach	3172	0.22	0.13	0.15	0.16
	3145		0.2	0.18	0.26
	3147		0.19	0.2	0.11
	3153		0.19	0.13	0.1
	3162		0.27	0.27	0.26

Table 8. continued

Strawberries	3201	0.21	0.15	0.16	0.09
	3190	0.27	0.24	0.26	0.24
	3186	0.27	0.18	0.19	0.13
	3211	0.26	0.16	0.19	0.12
	3221		0.23	0.15	0.12
Wheat	3287	0.15	0.18	0.2	0.2
	3270	0.15	0.14	0.17	0.1
	3260	0.21	0.22	0.21	0.23
	3261	0.19	0.18	0.14	0.15
	3259	0.21	0.25	0.17	0.23
Wheat Bran	3116	0.17	0.19	0.2	0.22
	3127	0.14		0.18	0.17
	3110	0.16	0.18	0.14	0.14
	3120	0.16	0.2	0.18	0.16
	3132	0.17	0.14	0.23	0.22
Zucchini	3291	0.3	0.22	0.23	0.2
	3288	0.23	0.16		0.24
	3276	0.16	0.17	0.23	0.18
	3257	0.16	0.16		0.22
	3262	0.19	0.16	0.24	0.21

Table 9. Micronucleated NCE frequencies measured over 44 days in mice fed vegetable, fruit or grain supplemented diets.

		MN-NCE Frequency (% per 1,000,000 total NCE)			
Treatment	**Tag #**	**Day 0**	**Day 14**	**Day 28**	**Day 44**
AIN-93G Control	3121	0.15	0.13	0.13	0.12
	3109	0.15	0.14	0.12	0.12
	3126	0.19	0.15	0.13	0.14
	3124	0.15	0.15	0.15	0.13
	3133	0.13	0.13	0.12	0.11
Apples	3170	0.12	0.11	0.1	0.1
	3142		0.14	0.14	0.13
	3157		0.12	0.13	0.12
	3152		0.12	0.11	0.1
	3163	0.12	0.12	0.1	0.1
Asparagus	3183	0.13		0.12	0.13
	3195	0.15	0.15	0.15	0.15
	3200	0.17	0.16	0.16	0.16
	3205	0.14	0.14	0.13	0.14
	3217	0.15	0.15	0.15	0.16
Barley	3187	0.11	0.1	0.09	0.09
	3189	0.14	0.12	0.13	0.13
	3219	0.15	0.13	0.13	0.13
	3204	0.14	0.13	0.13	0.13
	3207	0.13	0.14	0.12	0.14
Beets	3194	0.14	0.14	0.13	0.12
	3196		Tail Sliced		
	3215	0.14	0.14	0.12	0.16
	3208	0.12	0.11	0.11	0.13
	3202	0.14	0.13	0.12	0.13

Table 9. continued

Black Tea	3284	0.13	0.13	0.15	
	3283	0.14	0.15	0.15	0.15
	3275	0.14	0.15	0.15	0.13
	3258	0.14	0.14	0.21	0.17
	3271	0.17	0.18		0.16
Broccoli	3117	0.14	0.14	0.12	0.12
	3122	0.15	0.17	0.15	0.14
	3106	0.16	0.14	0.14	0.14
	3130	0.17	0.16	0.13	0.14
	3131	0.14	0.15	0.13	0.13
Brussel Sprouts	3173	0.16	0.14	0.14	0.14
	3141		0.11	0.1	0.12
	3154		0.14	0.13	0.14
	3150		0.12	0.11	0.12
	3169		0.15	0.14	0.15
Cabbage	3197	0.14	0.13	0.13	0.14
	3188	0.14	0.13	0.12	0.14
	3199	0.13	0.13	0.13	0.13
	3203	0.14	0.15	0.14	0.15
	3209	0.15	0.14	0.14	0.17
Canteloupe	3282	0.13	0.14	0.15	0.13
	3279	0.16	0.17		0.14
	3281	0.14	0.15	0.14	0.14
	3268	0.14	0.14	0.15	0.13
	3264	0.15	0.14	0.16	0.13

Table 9. continued

Cauliflower	3198	0.13	0.15	0.13	0.14
	3182	0.13	0.13	0.12	0.12
	3212	0.12	0.15	0.14	0.16
	3216	0.17	0.17	0.16	0.18
	3220	0.13	0.12	0.12	0.15
Carrots	3112	0.16	0.18	0.15	0.13
	3114	0.15	0.14	0.14	0.13
	3102	0.14	0.15	0.14	0.12
	3128	0.14	0.14	0.13	0.12
	3135	0.13	0.15	0.13	0.14
Corn	3174	0.14	0.12	0.11	0.12
	3140		0.13	0.13	0.13
	3156		0.13	0.13	0.14
	3159		0.13	0.12	0.13
	3167	0.14	0.11	0.1	0.1
Flaxseed	3101	0.14	0.14	0.12	0.12
	3125	0.14	0.14	0.13	0.11
	3129	0.17	0.13	0.11	0.11
	3113	0.14	0.16	0.14	0.14
	3107	0.14	0.13	0.12	0.12
Green Beans	3171	0.17	0.13	0.13	0.13
	3144	0.13	0.14	0.14	0.12
	3151		0.13	0.12	0.13
	3161		0.16	0.16	0.15
	3166	0.14	0.12	0.11	0.11

Table 9. continued

Green Peas	3123	0.15	0.15	0.15	0.13
	3105	0.13	0.12	0.12	0.14
	3103	0.14		0.14	0.12
	3118	0.14	0.14	0.13	0.12
	3111	0.14	0.14	0.14	0.14
Green Peppers	3192	0.15		0.12	0.14
	3185	0.15	0.11	0.12	0.12
	3206	0.16	0.15	0.15	0.15
	3218		Tail Sliced		
	3210	0.2	0.22	0.23	0.16
Green Tea	3289	0.14	0.13	0.15	0.14
	3278	0.14	0.13	0.15	
	3280	0.15	0.15		0.17
	3266	0.16	0.16		
	3263	0.14	0.14	0.15	0.15
Lentils	3143		0.14	0.14	0.13
	3148		0.1	0.11	0.09
	3155		Dead		
	3165	0.15	0.13	0.12	0.11
	3160	0.13	0.13	0.14	0.12
Oats	3290	0.14	0.14	0.14	0.13
	3273	0.15	0.14		0.15
	3274			0.15	0.14
	3267	0.13	0.15		0.13
	3269	0.15	0.16	0.15	0.13

Table 9. continued

Oranges	3108	0.15	0.15	0.14	0.12
	3104	0.14		0.13	0.13
	3115	0.15	0.14	0.13	0.12
	3119	0.13	0.12	0.16	0.12
	3134	0.13	0.15	0.14	0.13
Potato	3149		0.14	0.14	0.12
	3146		0.1	0.1	0.09
	3158	0.12	0.11	0.11	0.1
	3168	0.11	0.1	0.09	0.09
	3164		0.14	0.12	0.12
Rye	3184	0.13	0.12	0.11	0.12
	3191	0.11	0.11	0.1	0.11
	3193	0.15	0.16	0.16	0.15
	3213	0.12	0.1	0.11	0.12
	3214	0.12	0.12	0.09	0.11
Soybeans	3285	0.16	0.16	0.17	0.13
	3286	0.15	0.14	0.15	0.13
	3277	0.15	0.14		0.13
	3272	0.15	0.15	0.15	0.13
	3265	0.13	0.14	0.14	0.12
Spinach	3172	0.13	0.13	0.11	0.12
	3145		0.16	0.16	0.15
	3147		0.15	0.14	0.13
	3153		0.12	0.12	0.12
	3162		0.15	0.15	0.13

Table 9. continued

Strawberries	3201	0.13	0.12	0.11	0.1
	3190	0.18	0.17	0.16	0.16
	3186	0.14	0.14	0.12	0.13
	3211	0.15	0.14	0.13	0.12
	3221		0.13	0.13	0.14
Wheat	3287	0.15	0.13	0.15	0.13
	3270	0.14	0.13	0.13	0.12
	3260	0.15	0.15	0.15	0.14
	3261	0.14	0.13	0.14	0.13
	3259	0.14	0.15	0.15	0.13
Wheat Bran	3116	0.15	0.13	0.14	0.13
	3127	0.14		0.14	0.13
	3110	0.13	0.14	0.13	0.13
	3120	0.14	0.14	0.13	0.13
	3132	0.14	0.14	0.13	0.13
Zucchini	32[illegible]1	0.16	0.15	0.15	0.14
	3288	0.17	0.15		0.15
	3276	0.16	0.14	0.13	0.13
	3257	0.13	0.15		0.12
	3262	0.15	0.15	0.16	0.14

Table 10. Micronucleated reticulocyte frequencies measured over 44 days in a repeat study.

		MN-RET Frequency (% per 10,000 total reticulocytes)			
Treatment	**Tag#**	**Day 0**	**Day 14**	**Day 28**	**Day 44**
Lab Chow	3455	0.16	0.17	0.14	0.14
	3484	0.21	0.13	0.2	0.16
	3448	0.18	0.16	0.1	0.18
	3446	0.2		0.17	0.19
	3477	0.18	0.19	0.18	0.19
AIN-93G Control	3458	0.23	0.22	0.17	0.17
	3479	0.15		0.14	0.16
	3471	0.2	0.15	0.17	0.21
	3463	0.17	0.14	0.15	0.18
	3445		0.2	0.11	
	3465	0.21	0.26	0.16	0.24
	3469			0.23	0.23
	3482	0.22	0.19	0.27	0.2
	3450	0.15	0.2	0.18	0.14
	3478	0.2	0.22	0.23	0.19
Broccoli	3462	0.17		0.24	0.19
	3468	0.22	0.2	0.23	0.15
	3467	0.19	0.2	0.23	0.21
	3461	0.18		0.12	0.18
	3474	0.16		0.18	0.22

Table 10. continued

Flaxseed	3454	0.14	0.13	0.12	0.09
	3483	0.23	0.16	0.16	0.16
	3466	0.16		0.11	
	3456	0.18	0.11	0.1	0.17
	3452	0.16		0.15	0.16
Oranges	3464	0.2	0.25	0.2	0.23
	3460	0.16	0.22	0.11	0.15
	3470	0.18		0.13	0.16
	3472	0.22	0.22	0.14	0.23
	3447		0.19	0.22	0.17
Peas	3457	0.22	0.24	0.22	0.21
	3459		0.22	0.21	0.21
	3453	0.17	0.28	0.23	0.23
	3481			0.21	0.18
	3449			0.19	0.18
Wheat Bran	3476	0.2	0.19	0.13	0.14
	3475	0.21		0.18	0.13
	3451	0.12	0.12	0.16	0.17
	3473			0.24	0.24
	3480	0.17	0.16	0.18	0.17

Table 11. Micronucleated NCE frequencies measured over 44 days in a repeat study.

		MN-NCE Frequency (% per 1,000,000 total NCE)			
Treatment	**Tag #**	**Day 0**	**Day 14**	**Day 28**	**Day 44**
Lab Chow	3455	0.12	0.12	0.11	0.11
	3484	0.15	0.13	0.13	0.13
	3448	0.15	0.14	0.14	0.14
	3446	0.13		0.12	0.13
	3477	0.14	0.14	0.13	0.13
AIN-93G Control	3458	0.15	0.16	0.17	0.16
	3479	0.13		0.14	0.13
	3471	0.13	0.13	0.12	0.12
	3463	0.14	0.14	0.16	0.16
	3445		0.11	0.12	
	3465	0.16	0.16	0.15	0.14
	3469			0.13	0.13
	3482	0.15	0.17	0.16	0.15
	3450	0.13	0.13	0.13	0.12
	3478	0.15	0.14	0.15	0.15
Broccoli	3462	0.16		0.15	0.14
	3468	0.12	0.13	0.14	0.13
	3467	0.13	0.14	0.14	0.13
	3461	0.11		0.14	0.13
	3474	0.14		0.15	0.16

Table 11. continued

Flaxseed	3454	0.12	0.13	0.12	0.11
	3483	0.15	0.13	0.13	0.13
	3466	0.11		0.1	
	3456	0.15	0.13	0.14	0.13
	3452	0.13		0.14	0.14
Oranges	3464	0.13	0.15	0.14	0.14
	3460	0.13	0.13	0.13	0.14
	3470	0.13		0.14	0.13
	3472	0.14	0.15	0.14	0.15
	3447		0.12	0.13	0.12
Peas	3457	0.15	0.17	0.17	0.15
	3459		0.16	0.16	0.15
	3453	0.14	0.14	0.16	0.16
	3481			0.16	0.15
	3449			0.13	0.13
Wheat Bran	3476	0.14	0.16	0.17	0.15
	3475	0.13		0.13	0.13
	3451	0.13	0.12	0.13	0.12
	3473			0.18	0.19
	3480	0.12	0.12	0.12	0.12

Table 12. Micronucleated reticulocyte frequencies in normal, sham and splenectomized mice fed flaxseed supplemented diets.

		MN-RET Frequency (% per 10,000 total reticulocytes)	
Treatment	**Tag#**	**Day 0**	**Day 44**
Normal	3572	0.22	0.17
	3581	0.15	0.26
	3591	0.18	0.15
	3600		0.17
	3605	0.22	0.24
	3570	0.27	0.22
	3576	0.18	0.19
	3585	0.25	0.1
	3592	0.13	0.16
	3588	0.26	
Sham	3579	0.22	0.11
	3604	0.14	0.08
	3602	0.18	0.18
	3583	0.17	0.16
	3587	0.2	0.16
	3573	0.16	0.1
	3578	0.21	0.18
	3598	0.12	
	3594	0.27	0.16
	3582	0.16	0.16
	3580	0.25	0.15
	3593	0.23	0.13
	3569		0.16

Table 12. continued

Splenectomized	3599	0.17	0.12
	3603		0.16
	3589	0.12	0.22
	3597	0.15	0.06
	3584	0.21	0.21
	3595	0.25	0.16
	3601	0.2	
	3577	0.26	0.19
	3586	0.22	0.21
	3590	0.18	0.13
	3571	0.22	0.18
	3596	0.17	0.16
	3575	0.2	0.19

Table 13. Micronucleated NCE frequencies in normal, sham and splenectomized mice fed flaxseed supplemented diets.

		MN-NCE Frequency (% per 1,000,000 NCE)	
Treatment	**Tag#**	**Day 0**	**Day 44**
Normal	3572	0.15	0.1
	3581	0.15	0.11
	3591	0.15	0.11
	3600		0.12
	3605	0.15	0.12
	3570	0.14	0.12
	3576	0.16	0.13
	3585	0.14	0.11
	3592	0.13	0.13
	3588	0.13	
Sham	3579	0.11	0.09
	3604	0.12	0.09
	3602	0.15	0.13
	3583	0.12	0.1
	3587	0.13	0.11
	3573	0.14	0.12
	3578	0.14	0.13
	3598	0.13	
	3594	0.13	0.11
	3582	0.13	0.11
	3580	0.17	0.13
	3593	0.13	0.11
	3569		0.11

Table 13. continued

Splenectomized	3599	0.15	0.14
	3603		0.13
	3589	0.15	0.14
	3597	0.14	0.14
	3584	0.16	0.13
	3595	0.17	0.15
	3601	0.15	
	3577	0.16	0.13
	3586	0.17	0.13
	3590	0.16	0.13
	3571	0.16	0.13
	3596	0.14	0.12
	3575	0.17	0.15

Table 14. Spontaneous *lacZ* mutant frequencies in the colon of 3 week-old mice fed supplemented diets.

Treatment	Tag#	Total # of Plaques	# of Mutants	Mutant Frequency (x10-5)
Control AIN-93G	3633	107,500	4	3.7
	3634	240,000	8	3.3
	3642	125,000	4	3.2
	3643	150,000	4	2.7
	3673	375,000	7	1.9
	3674	427,500	14	3.3
	3817	160,000	11	6.9
	3819	650,000	26	4.0
	3820	582,500	8	1.4
	3823	330,000	17	5.2
	3969	437,500	5	1.1
	3970	412,500	7	1.7
	3971	495,000	18	3.6
	3972	570,000	12	2.1
	NT	355,000	8	2.2
	1	290,000	6	2.1
	3	237,500	9	3.8
	4B	165,000	1	0.6
	6B	375,000	6	1.6
	7B	227,500	4	1.8
Control with 9% Fat	3791	432,500	13	3.0
	3796	167,500	4	2.4
	3797	505,000	7	1.4
	3800	185,000	3	1.6
	3802	477,500	5	1.1
Broccoli	3607	60,000	3	5.0
	3616	347,500	9	2.6
	3811	142,500	10	7.0
	3815	110,000	5	4.5
	3827	67,500	4	5.9
Carrots	3608	17,500	1	5.7
	3610	132,500	6	4.5
	3666	45,000	1	2.2
	3875	202,500	4	2.0

Table 14. continued

Oranges	3609	12,500	1	8.0
	3617	10,000	3	3.0
	3701	30,000	5	16.7
	3707	125,000	3	2.4
	3714	185,000	6	3.2
Flaxseed	3804	302,500	6	2.0
	3805	242,500	6	2.5
	3958	427,500	8	1.9
	3959	712,000	14	2.0
	3960	527,500	19	3.6
	3962	445,000	12	2.7
	54009	310,000	5	1.6
	54015	417,500	14	3.4
	54017	515,000	15	2.9
Cabbage	3863	87,500	2	2.3
	3868	207,500	8	3.8
	3902	220,000	6	2.7
	3903	200,000	3	1.5
	3950	132,500	3	2.3
Green Peppers	3917	355,000	8	2.3
	3920	240,000	9	3.8
	3924	182,500	2	1.1
	3965	85,000	2	2.4
	3966	200,000	7	3.5
Green Peas	3908	90,000	6	6.7
	3911	105,000	3	2.9
	3936	272,500	6	2.2
	3938	140,000	8	5.7
	3940	227,500	11	4.8
	3941	157,000	3	1.9
Strawberries	54001	257,500	10	3.9
	54012	147,500	7	4.7
	54016	147,500	4	2.71
	54027	112,500	1	0.9
	54028	60,000	2	3.33
	54052	117,500	3	2.6

Table 15. Spontaneous *lacZ* mutant frequencies in the colon of 8 week-old mice fed supplemented diets.

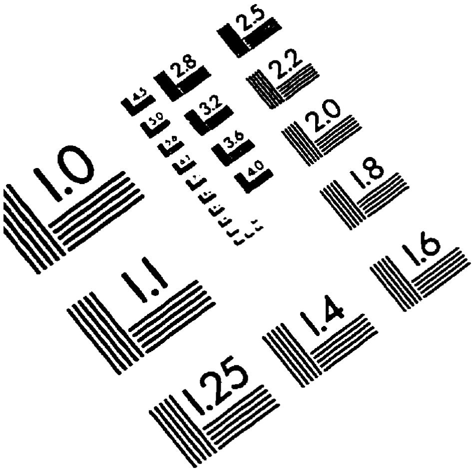
Treatment	Tag#	Total # of Plaques	# of Mutants	Mutant Frequency (x10-5)
Control AIN-93G	3670	255,000	12	4.71
	3676	195,000	7	3.59
	3818	230,000	9	3.91
	3822	467,500	15	3.21
	3864	447,500	13	2.91
	3865	617,500	28	4.53
	3967	700,000	19	2.71
	3968	665,000	18	2.71
Control with 9% Fat	3698	517,500	19	3.67
	3717	442,500	17	3.84
	3762	432,500	12	2.78
	3793	537,500	14	2.61
	3794	140,000	5	3.57
	3799	617,500	25	4.05
Flaxseed	3806	405,000	17	4.2
	3957	617,500	23	3.73
	3961	605,000	58	9.59
	3963	492,500	18	3.65
	3964	585,000	27	4.62
Green Peppers	3923	392,500	3	0.76
	3925	230,000	7	3.04
	3926	195,000	3	1.54
	3928	315,000	6	1.9
	3929	55,000	1	1.82
	3930	220,000	6	2.73

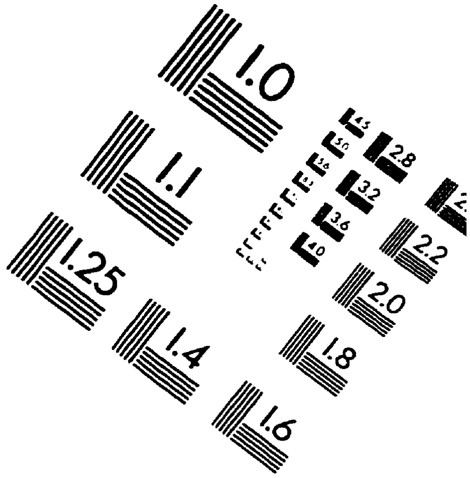
TABLE 16. Spontaneous *lacZ* mutant frequencies in the small intestine for 2 and 4 week-old mice maintained on lab chow during development

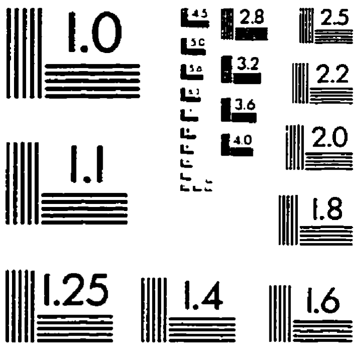
Treatment	Tag#	Age (wks)	Total # of Plaques	# of Mutants	Mutant Frequency (x10-5)
Orginal Data**					
Lab Chow	2251D	2	39,700	2	5.0
	2251E	2	80,000	4	5.0
	2253D	2	107,700	4	3.7
	2253E	2	189,050	7	3.7
	61995	2	114,950	9	7.8
	2255E	2	246,700	7	2.8
	2255F	2	83,750	6	7.2
	2251F	4	365,550	8	2.2
	2252F	4	130,000	5	3.9
	2253E	4	602,500	38	3.3
	2253F	4	585,100	22	3.8
	61995E	4	69,150	7	10.1
	2252G	4	433,600	35	8.1
Repackaged Data					
Lab Chow	2251D	2	87,500	6	6.9
	2251E	2	247,500	9	3.6
	2253D	2	130,000	4	3.1
	2253E	2	162,500	14	8.6
	2255E	2	55,000	6	10.9
	2255F	2	42,500	5	11.8
	61995	2	82,500	6	7.3
	2251F	4	100,000	3	3.0
	2252F	4	115,000	6	5.2
	2253F	4	277,500	13	4.7
	61995E	4	57,500	7	12.2

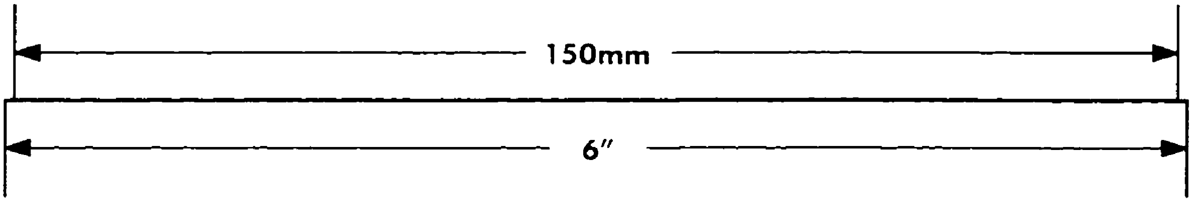
** original data from Paashius-Lew and Heddle, 1997

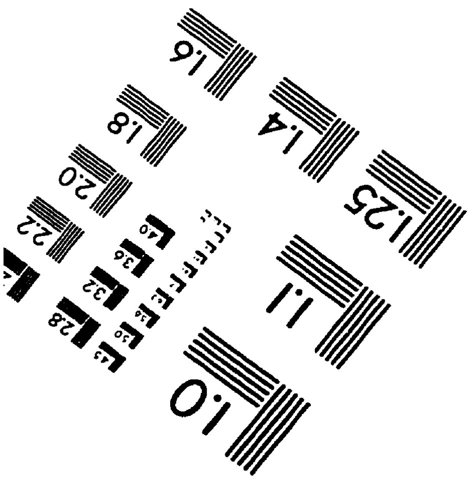
IMAGE EVALUATION TEST TARGET (QA-3)

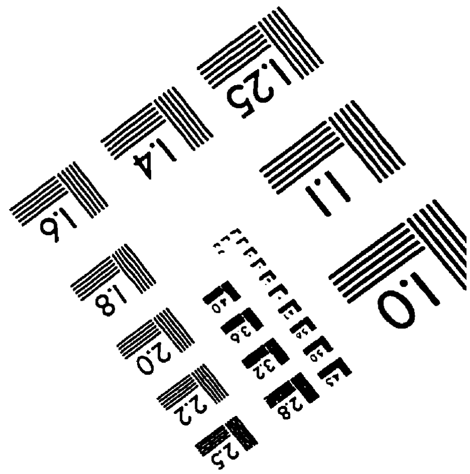












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