The Applicability of a Short-term Test for Detection of Modifying Effects of Dietary Factors in Rodent Colon Carcinogenesis

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THE APPLICABILITY OF A SHORT-TERM TEST FOR DETECTION OF MODIFYING EFFECTS OF DIETARY FACTORS IN RODENT COLON CARCINOGENESIS

Ph.D. Thesis

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DATA SHEET

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Abstract: The present studies were initiated to develop a short-term rodent model to assess the influence of different dietary components on the development of colon cancer.
Diets with different dietary components, i.e. dietary fibre, fat, sucrose, and starches were tested in male rats initiated with DMH-2HCl or AOM for their modulating effect on the development of aberrant crypt foci (ACF). Furthermore the heterocyclic amines IQ and PhIP were introduced in the assay as inducers of ACF in mice and rats and their role in colon carcinogenesis in mice was investigated.
ACF were found to be induced in rodent colon by the colon carcinogens DMH-2HCl, AOM, IQ, and PhIP and it was shown that the incidence of the induced ACF could be modulated by dietary components such as sucrose, dietary fibre, and starch.

Key words: Aberrant crypt foci, ACF, colon cancer, dietary modulators, DMH-2HCl, AOM, IQ, PhIP, rat, mice.

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Front cover: Illustration of the suggested relation between normal crypts, aberrant crypts, ACF, adenomas and adenocarcinomas.
1: Normal crypts, 2: Aberrant crypt focus, 3: Adenoma,
4: Adenocarcinomas.
Drawn by Bent Vismann

PREFACE

The present studies constitute part of the research on the relation between diet and cancer at the Danish Veterinary and Food Administration.

The aim of the studies presented here was to develop a short-term animal model to assess the influence of different dietary components on the development of colon cancer. The biomarker selected for the short-term assay should be a putative preneoplastic change that took place in the colon mucosa prior to the development of recognizable colon cancer, and hence should provide an alternative to the testing of dietary modulators without using cancer as endpoint.

I wish to thank professor Ole Andersen, Institute of Life Sciences and Chemistry, Roskilde University Center, Denmark, for being my supervisor and for his interest and fruitful comments during the writing phase. Otto Meyer, Head of Biological Section, Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, is thanked for being my adviser, and, together with Inger Thorup, DVM, are thanked for many stimulating years of collaboration in the field of colon cancer research.
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Søborg, August 1997

Eva Kristiansen

CONTENTS

SUMMARY

Colon cancer is one of the most frequent types of cancer in humans, and epidemiological studies have indicated that the incidence of colon cancer is linked, in large measure, to the composition of the diet.

Many of the alterations seen in human colonic mucosa during colon cancer development are also found in animal models. The cost and duration of traditional long-term animal studies with the endpoint cancer makes the screening for dietary modulators of colon cancer almost infinite. Therefore, short-term bioassays are recognized as being increasingly vital to this screening process.

The presented studies were initiated to find an intermediate biomarker which could be used in a short-term assay for screening of dietary modulators of colon carcinogenesis.

The intermediate biomarker chosen for the studies was a histological biomarker, the aberrant crypt foci (ACF). ACF are morphological lesions identified topographically in intact Giemsa stained colons, without being embedded in paraffin and sectioned.

Different diets, high in fat, dietary fibre, starches or sucrose were investigated in the ACF assay in male rats initiated with the model colon carcinogens 1,2-dimethylhydrazine dihydrochloride (DMH-2HCl) or azoxymethane (AOM). A modulating effect on the DMH-2HCl- or AOM-induced ACF was detected with a dietary fibre, starch and sucrose. In general the most consistent finding was an effect on the total number of ACF and the number of small (1-3 crypt/focus) ACF. The studies which proceeded to tumour development did not show a clear relation between the outcome of ACF and tumours with any of the dietary components tested.

The heterocyclic amines, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), were found to be able to induce ACF in both mice and rats, and in rats the incidence of ACF could be modulated by a diet high in sucrose. The effect was, like for DMH-2HCl and AOM, seen in the total number of ACF and the number of small (1-3 crypt/focus) ACF. Even though IQ and PhIP are not known to be colon carcinogens in mice, ACF were detected in mice after both short-time and long-time exposure to IQ or PhIP. The number of ACF increased over time, and at all time-points the numbers of small ACF were predominant.

It was shown that ACF are induced in rodent colon by colon carcinogens such as DMH-2HCl, AOM, IQ, and PhIP and that the incidence of induced ACF could be modulated by different dietary components. As no clear relation between the outcome of ACF and tumours was found in the studies, the results could indicate that ACF and colon tumours represent two parallel

independent events as a consequence of the cancer initiation, i.e. the ACF not being preneoplastic per se. An alternative explanation could be that only some of the ACF are precursor lesions for colon tumours and that the time point in the colon carcinogenic process, at which the ACF are scored is not the ideal to find a true relation between ACF and colon tumours.

The question whether ACF represent true preneoplastic lesions is still open. A better understanding of the early detected lesions in colon carcinogenesis in animal models is needed, before the presence of ACF can be interpreted as a reliable indicator of a later development of cancer, and the ACF being predictive of a human colon cancer potential.

SUMMARY IN DANISH (SAMMENDRAG)

Tyktarmskræft er en af de hyppigste kræftformer hos mennesket, og befolkningsundersøgelser tyder på, at hyppigheden af tyktarmskræft i høj grad er knyttet til kostens sammensætning.

Mange af de forandringer, der ses i tarmslimhinden under udviklingen af kræft hos mennesker, ses også i dyremodeller. Udgifterne og varigheden af traditionelle langtidsdyreforsøg med endepunktet kræft gør screeningen for kostrelaterede modulatorer næsten uendelig. Korttidsundersøgelser er derfor blevet tiltagende vigtige for denne screeningsproces.

Nærværende undersøgelser blev igangsat med henblik på at finde en intermediær biomarkør, som kunne bruges i en korttidstest for screening af kostrelaterede modulatorer af tyktarmskræftudviklingen.

Den intermediære biomarkør, der blev valgt til undersøgelserne, var en histologisk biomarkør, "aberrant crypt foci" (ACF). ACF er morfologiske forandringer, som påvises topografisk i Giemsafarvet tyktarm, uden at denne er indstøbt i paraffin og snittet.

Forskellige fodertyper med højt indhold af fedt, kostfiber, stivelse eller sukker blev undersøgt i ACF-testen i hanrotter initieret med de tyktarmskræftfremkaldende stoffer 1,2-dimethylhydrazindihydrochlorid (DMH-2HCl) eller azoxymethan (AOM). Kostfiber, stivelse og sukker modulerede de DMH-2HCl- eller AOM-inducerede ACF. Det generelle fund var en effekt på det totale antal ACF og på antallet af små ACF (1-3 krypter/focus). De undersøgelser, der fortsatte til udviklingen af tumorer, viste ingen klar sammenhæng mellem mængden af ACF og tumorer og de fodertyper, der blev undersøgt.

De heterocycliske aminer, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) og 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), inducerede ACF hos både mus og rotter, og hos rotter kunne mængden af ACF moduleres af et foder med et højt indhold af sukker. Effekten sås, ligesom ved DMH-2HCl og AOM, på det totale antal ACF og på antallet af små ACF (1-3 krypter/focus). Selv om IQ og PhIP ikke er kendt for at give tyktarmskræft hos mus, blev der påvist ACF hos mus såvel efter kort som lang tids udsættelse for IQ eller PhIP. ACF-antallet øgedes med tiden, og på alle målte tidspunkter var små ACF de dominerende.

Forsøgene viste, at ACF induceres i gnavertyktarm af stoffer, der giver tyktarmskræft, såsom DMH-2HCl, AOM, IQ og PhIP, og at incidensen af inducerede ACF kan moduleres af forskellige kostkomponenter. Da der ikke blev fundet en klar sammenhæng mellem mængden af ACF og tumorer, kunne resultaterne indicere at ACF og tyktarmskræft repræsenterer to parallelle, uafhængige hændelser som en konsekvens af kræftinitieringen,

dvs. ACF er i sig selv ikke forstadier til kræft. En alternativ forklaring kunne være, at det kun er visse ACF, der er forstadier til tyktarmskræft, og at det tidspunkt i tyktarmskræftprocessen ved hvilken ACF scores, ikke er det ideelle for at finde en sand sammenhæng mellem de preneoplastiske ACF og tyktarmstumorer.

Spørgsmålet, om ACF er sande forstadier til kræft, er stadig uafklaret. Der er behov for en bedre indsigt i de tidligt påviste skader i udviklingen af tyktarmskræft i dyremodeller, før tilstedeværelsen af ACF kan tolkes som en troværdig indikator på senere udvikling af kræft, og dermed forudsige mulig human tyktarmskræft.

LIST OF PUBLICATIONS

The publications included in this thesis are:

Paper I — Inger Thorup, Otto Meyer, and Eva Kristiansen (1994) Influence of a dietary fiber on development of dimethylhydrazine-induced aberrant crypt foci and colon tumor incidence in Wistar rats. Nutr. Cancer 21, 177-182.

Paper II — Eva Kristiansen, Inger Thorup, and Otto Meyer (1995) Influence of different diets on development of DMH-induced aberrant crypt foci and colon tumor incidence in Wistar rats. Nutr. Cancer 23, 151-159.

Paper III — Inger Thorup, Otto Meyer and Eva Kristiansen (1995) Effect of potato starch, cornstarch, and sucrose on aberrant crypt foci in rats exposed to azoxymethane. Anticancer Res. 15, 2101-2106.

Paper IV — Eva Kristiansen, Otto Meyer, Inger Thorup (1997) The ability of two cooked food mutagens to induce aberrant crypt foci in mice. Eur. J. Cancer Prev. 6, 53-57.

Paper V — Eva Kristiansen, Otto Meyer, Inger Thorup (1996) Refined carbohydrate enhancement of aberrant crypt foci (ACF) in rat colon induced by the food-borne carcinogen 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ). Cancer Lett. 105, 147-151.

Paper VI — Eva Kristiansen (1996) The role of aberrant crypt foci induced by the two heterocyclic amines 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) in the development of colon cancer in mice. Cancer Lett. 110, 187-192.

Papers related to the aberrant crypt foci research not included in this thesis.

Inger Thorup, Otto Meyer, and Eva Kristiansen (1992) Effect of a dietary fiber (beet fiber) on dimethylhydrazine-induced colon cancer in Wistar rats. Nutr. Cancer 17, 251-261.

Sørensen IK, Kristiansen E, Mortensen A, van Kranen H, van Kreijl C, Fodde R, and Thorgeirsson SS (1997) Short-term carcinogenicity testing of a potent murine intestinal mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), in *Apc*1638N transgenic mice. Carcinogenesis, 18, 777-781.

ABBREVIATIONS

AαC:	2-amino-9*H*-pyrido[2,3*b*]indole
ACF:	aberrant crypt focus/foci
AOM:	azoxymethane
APC:	adenomatous polyposis coli
CHL:	chlorophyllin
DMH-2HCl:	1,2-dimethylhydrazine dihydrochloride
EGF-R:	epidermal growth factor receptor
FAP:	familial adenomatous polyposis
IQ:	2-amino-3-methyl-imidazo[4,5-*f*]quinoline
I3C:	indole-3-carbinol
MAM:	methyl azoxymethanol acetate
MeIQx:	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
NA:	nuclear aberrations
ODC:	ornithine decarboxylase
PhIP:	2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine
PCNA:	proliferating cell nuclear antigen
SEM:	scanning electron microscopy
TGFα:	transforming growth factor α

INTRODUCTION

Epidemiology of colon cancer

During the past decades, epidemiological studies have indicated that colon cancer in humans is induced, in large measure, by substances in our environment. Comparative studies of geographical variations in the incidence of colon cancer indicate a 10-20 fold difference in incidence between the country with the highest and the country with the lowest rate. This variation in incidence suggests a large potential for prevention of colon cancer (American Cancer Society 1995, Muir et al. 1987). Likewise, in 1981, Doll & Peto estimated that 90% of colon cancers in USA could be linked to the diet. This estimate has recently been reduced to 70% (range 50-80%) by WC Willet (Nelson 1996) because of clear evidence since 1981 that physical activity plays an important protective role in colon cancer.

Colon cancer is the third most frequent cancer in the world in both sexes, after cancers of the lung and stomach in males and after those of breast and cervix in females (Coleman et al. 1993).

In 1993, the annual number of newly diagnosed cases of colorectal cancers in Denmark was approximately 3300 (Storm et al. 1996). The incidence has increased since 1960, and the average annual numbers of deaths in Denmark due to cancer of the colon in 1983-1987 was 547 for males and 651 for females (Engeland et al. 1995).

The intestinal mucosa represents one of the largest interfaces between the human body and the external environment. It is exposed to a variety of ingested carcinogens and to many compounds that may be converted to carcinogens by the microflora and the environment within the intestinal ecosystem.

The question whether certain dietary factors could be involved in colon cancer has important public health implications, and several hypotheses have been put forward concerning the factors responsible for the differences in the international incidences. So far the epidemiological data indicate that the dietary intake of meat, protein, and fat are consistently positively related to risk, while vegetable consumption and probably also foods high in fibre are inversely associated with risk (Willett 1989, Potter et al. 1993).

Because of the inherent weakness and limitations in epidemiological studies, laboratory animal models are necessary to support and further explain the findings in humans.

Experimental colon cancer

Laboratory rodents have been used for long time to predict carcinogenic risk in humans. The basis upon which the extrapolation from one species to another lies is the similarity among animals and humans at the cellular levels (Calabrese 1983). The relevance of rodent studies, with short life spans and exposures to high doses of chemical substances, as models for human disease processes is sometimes questioned. However, the observation of many of the same alterations in human colonic mucosa as in these animal models lends some credence to animal models. Although spontaneous tumours of the intestine are uncommon in animals, rodents readily develop adenomas and carcinomas when exposed to various chemical substances (Shamsuddin 1983, Rogers & Nauss 1985a, Goldin 1988). Despite the lack of sufficient evidence that these chemicals are human intestinal carcinogens, the chemically induced tumours resemble the colon tumours seen in humans and thus provide an opportunity to study the effects of diets, chemopreventive substances and other factors in tumorigenesis (Freeman 1983, Jacobs 1983, Reddy 1983, Maskens 1983, Ward & Ohshima 1985, Greene et al. 1987).

Short-term assay for colon cancer

The aim of the studies presented was to develop and validate a short-term animal model to assess the influence of different dietary components on the development of colon cancer. The use of traditional long-term animal studies makes this job almost endless because of their duration and cost and due to the plethora of dietary components of interest. Therefore, short-term *in vivo* bioassays are recognized as being increasingly vital in the screening process. Thus, we looked for an intermediate biomarker which should be recognized as a "putative preneoplastic change" that takes place in colonic mucosa prior to the development of recognizable malignant disease (colon cancer). Biomarkers for colon cancer, if validated, may provide an alternative to the traditional testing of dietary modulators using cancer as an endpoint.

The putative premalignant changes that appear to precede colon cancer, and which could be used in a short-term assay for colon cancer, include biochemical, molecular, and morphological alterations. Many of these changes have been observed in both laboratory animals dosed with colon carcinogens and in human colonic mucosa at increased risk for cancer.

The applicability of short term biomarkers are based upon a number of criteria. They should:

- be modulated in frequency by dietary components known to influence colon cancer outcome
- have a short "latency time" in terms of weeks
- be able to be assessed in a simple, reliable and quantitative manner
- occur at a frequency high enough to allow for their biological and statistical evaluation relevant to cancer

Since carcinogenesis in colon is recognized to include multiple genetic events (Vogelstein et al. 1988, Kinzler & Vogelstein 1992), the use of single biomarkers can be difficult, as they might relate to and be recognizable at only one or a few of the many possible stages.

The intermediate biomarker chosen in the presented studies is a histological biomarker, the aberrant crypt foci (ACF) (Bird 1987). ACF are defined as morphological lesions identified topographically in intact colons, i.e. not embedded in paraffin and sectioned, and thus not defined by their histological features (Bird and Pretlow 1992).

One advantage of this short-term bioassay is that the ACF scored as biomarkers subsequently can be isolated by microdissection and further analyzed by other methods applicable to fixed tissue. In this way, ACF have been identified as having certain characteristics in common with colonic tumours in rodents and humans. ACF have been characterized by their histological features in terms of grade of dysplasia (McLellan et al. 1991a, Roncucci et al. 1991, Pretlow et al. 1992) and proliferative activity (McLellan et al. 1991b, Pretlow et al. 1994a, Roncucci et al. 1993). Some of the genetic alterations known to be involved in colon carcinogenesis (Vogelstein et al. 1988, Kinzler & Vogelstein 1992), have also been detected in ACF. Thus K-*ras* mutations have been identified in ACF of mice (Zaidi et al. 1995), rats (Shivapurkar et al. 1994, Stopera & Bird 1992, Stopera et al. 1992b, Tachino et al. 1995), and humans (Pretlow et al. 1993, Smith et al. 1994, Jen et al. 1994, Yamashita et al. 1995), and *APC* mutations have been identified in humans (Smith et al. 1994, Jen et al. 1994). Over-expression of carcinoembryonic antigen has been detected in human ACF (Pretlow et al. 1994b), and in rat colon the expression of *c-fos* (Stopera et al. 1992a) is significantly increased in the ACF. Also the levels of proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGF-R), transforming growth factor α (TGFα), and ornithine decarboxylase (ODC) have been found to be increased in ACF isolated from rat colon (Berger et al. 1994). The ACF assay is continuously evaluated by the extensive research conducted up to now (more than 200 papers on the assay since 1987), and the assay is routinely used in the screening program for evaluation of potential chemopreventive agents by the National Cancer Institute chemoprevention drug development program (Steele at al. 1994).

The ACF identified in the different studies which are included in the present thesis have been characterized further in many parameters (Thorup 1997). However, this thesis includes only the morphological features of the ACF in the intact unembedded rodent colon.

DESCRIPTION OF THE SHORT-TERM ASSAY

Normal colon crypts

The normal colon mucosa contains numerous test-tube shaped glands, the crypts of Lieberkühn, of which the single crypt is the unit structure of the colon. The crypts are open at the mucosal surface and have a closed base.

The balance between dividing cells in the deepest third of the crypts and migration and exfoliation at the surface maintains the flat-appearing surface of the colon (Deschner 1990). The cell population turnover time, defined as the time necessary to replace the number of cells present in the entire crypt, is reported to be 10 days for rat colon (Bertalanffy 1960) and 3 days for mouse colon (Oehlert & Büchner 1961).

Estimates of the total number of crypts/colon is 405.000 in the rat and 690.000 in the mouse (Deschner 1990), or 300 to 1000 similar crypts covering each square millimetre of the colon surface (Bruce 1990). The surface area of the individual crypt does not reveal any change from the proximal to the distal part of the rat colon. However, the number of crypts decreases markedly along the proximal-to-distal axis due to an increase in the epithelial surface between the crypts (Gutschmidt et al. 1983).

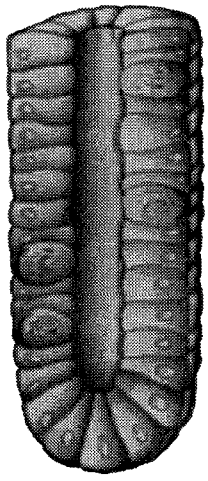

Figure 1. Normal colonic crypt.

Colon cancer inducers

1,2-dimethylhydrazine dihydrochloride (DMH-2HCl) and azoxymethane (AOM).

DMH-2HCl and AOM have proven to be of great value in experimental colon carcinogenesis studies due to their reliable and specific ability to produce colon tumours in several rodent species. Accordingly, they are the most widely used carcinogens for studying colon carcinogenesis in rodents.

DMH-2HCl is activated by metabolism via AOM and methyl azoxymethanol acetate (MAM) to yield the ultimate carcinogen. Both AOM and MAM are more effective than DMH-2HCl on a molar basis. Under physiological conditions, MAM is unstable and breaks down to form the strong alkylating agent, the methyldiazonium ion. The carcinogenicity of DMH-2HCl and AOM, is not definitively known for humans (Jacobs 1983).

2-amino-3-methyl-imidazo[4,5-f]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP).

IQ and PhIP belong to the group of heterocyclic amines which are reaction products of proteins that are formed during the heating (cooking, broiling, frying or grilling) of protein-containing foods. Both IQ and PhIP are mutagenic and carcinogenic in rodents (Eisenbrand & Tang 1993). IQ and PhIP are major mutagens present in heated fish and meat to which humans are regularly exposed, and epidemiological studies, although not definitive, are supportive of an association between dietary heterocyclic amine intake and risk of human colorectal cancer (Adamson et al. 1996).

IQ and PhIP are carcinogenic to the rat colon (Takayama et al. 1984a, Ito et al. 1991). Induction of colonic tumours have not been reported with IQ or PhIP in mice (Ohgaki et al. 1984a, Esumi et al. 1989, Kristiansen et al. 1997), but formation of ACF has been seen in mice after both IQ or PhIP administration (Tudek et al. 1989), and IQ has been shown to induce nuclear aberrations (NA) (i.e. micronuclei, karyorrhexis, and pyknosis (apoptotic bodies)) in mice colonic crypts (Bird & Bruce 1984, Dolara et al. 1986). As nuclear aberrations are cell-lethal events, the induction of NA suggests that IQ, or its active metabolite, is capable of reaching the colonic epithelium and exerting nucleotoxic effects. The mechanism behind IQ and PhIP induction of colonic lesions (ACF, NA) in mice is not known. Unchanged IQ is known to be present in the gut of mice after oral administration, and activation of this to a direct-acting genotoxin by the colonic microflora may thus lead to DNA damage in the colonic mucosa (Alldrick & Rowland 1988).

Carcinogen-induced changes in the crypts

It is generally believed that colon carcinogenesis, irrespective of animal species including man, is a focal and sequential process originating from a single crypt. The patterns of cellular alterations seen in colon during cancer development are remarkably similar with different chemical carcinogens. Probably, a neoplastic transformation of a small fraction of the epithelial cells in the colonic crypt is one of the initial steps in carcinogen-induced changes. It is very difficult to identify isolated neoplastic transformed cells in single crypts. Only when a crypt is partly or entirely populated by cytologically atypical cells it can be identified from the normal crypts. The atypical cells are generally characterized by their cytoplasmic basophilia, loss of goblet cells, a high nuclear-cytoplasmic ratio, prominent nucleoli, loss of cell polarity and most importantly an increase in proliferative activity (Chang 1984). Such changes have been detected histologically after four to nine weekly injections with DMH-2HCl or AOM (Chang 1984, Shamsuddin & Trump 1981a, Nakamura & Kino 1982).

Aberrant colon crypts

Aberrant crypt foci are distinct morphological lesions readily identifiable microscopically from the mucosal surface at a magnification of x 40 in unembedded colon tissue stained with methylene blue (Bird 1987). The methodology for identifying these lesions is simple. The colons of rats or mice treated with a colon carcinogen, are excised, cut open along the longitudinal axis and rinsed in 0.9% NaCl solution. The colons are then pinned on a cork slab and fixed in 4% neutral buffered formaldehyde. After fixation, methylene blue or Giemsa stain is used to visualize the ACF. The tissue is placed luminal side up in a petri dish with sufficient phosphate-buffered saline to cover the tissue.

As defined by McLellan & Bird (1988b), these aberrant crypts are larger than the normal crypts, have a thickened epithelial layer, frequently have a slit-shaped rather that round lumina, and have an increased pericryptal zone that separates them from the adjacent normal crypts. The parameters used to characterize the ACF are: total number of ACF in a colon and number of aberrant crypts per focus (crypt multiplicity). The ACF are often grouped into small, medium, large and extra large foci, according to the number of crypts per focus. The classification into the individual groups differs among investigators. The average number of ACF per colon, total ACF as well as the different groupings, are then compared in the treated and control groups. The distribution of ACF along the colon sometimes is recorded as well.

The ACF identified by Bird (1987) with light microscopy of methylene blue-stained whole-mount colon appear similar to lesions described earlier with scanning electron microscopy (SEM) in rats treated with colon carcinogens (Barkla & Tutton 1977, Karlin et al. 1977, Cooke et al. 1984). Likewise, by evaluating histological sections from rats treated with DMH-2HCl, Sunter et

al. (1978), Carachi et al. (1980) and Kimura et al. (1984), identified lesions with the same characteristics as ACF. In grossly normal colonic mucosa from patients with sporadic colon cancer similar lesions have been identified histologically (Oohara et al. 1980, Shamsuddin et al. 1981b). Compared to Bird's technique where the entire colon of a rodent can be evaluated in less than half an hour, the SEM only evaluate approximately 1 cm^2 of the colon and the analysis of lesions similar to ACF in histological sections would require thousands to millions of slides per animal and thus: "identifying the early precursor lesions by examining histological sections is analogous to looking for a needle in a haystack" (McLellan & Bird 1991). Thus, the ACF assay described by Bird (1987) has not identified new putative precursor stages in colon cancer, but it has made it possible to examine the entire colonic mucosa of a rodent or large areas of human colon in a relatively short period of time.

ACF are induced by colon carcinogens such as DMH-2HCl and AOM in a dose-dependent manner in rats and mice (McLellan & Bird 1988a, 1988b, Tudek et al. 1989, Bird et al. 1989, McLellan et al. 1991b), appear as early as two weeks after a single dose of carcinogen (McLellan & Bird 1988b), persist until the development of overt tumours (McLellan et al. 1991a, Pretlow et al. 1992a), and are rarely induced by carcinogens targeting other organs (McLellan & Bird 1988a, Bird et al. 1989, Tudek et al. 1989). Thus, the formation of ACF is suggestive of a specific response of the colon to colon carcinogens. Two to four weeks after the first carcinogen treatment the ACF consist of one to three crypts (Bird 1987, McLellan & Bird 1988b, Pretlow et al. 1992a), and with increasing time some of these foci become larger (McLellan & Bird 1988b, Bird et al. 1989, Pretlow et al. 1992a). Even though the number of ACF is dose dependent, this appears not to be the case for the number of crypts per focus (McLellan et al. 1991b). The size of the ACF can vary within the same animal. As illustrated by photomicrographs, foci of aberrant crypts composed of 2 to >20 crypts from one mouse have been presented by McLellan & Bird (1988b), and in a single microscopic field (magnification x 20) Bird et al. (1989) showed foci composed of 2 to 13 crypts in a rat. Nuclear features of dysplasia seem to be present in only a minority of ACF (McLellan et al. 1991a).

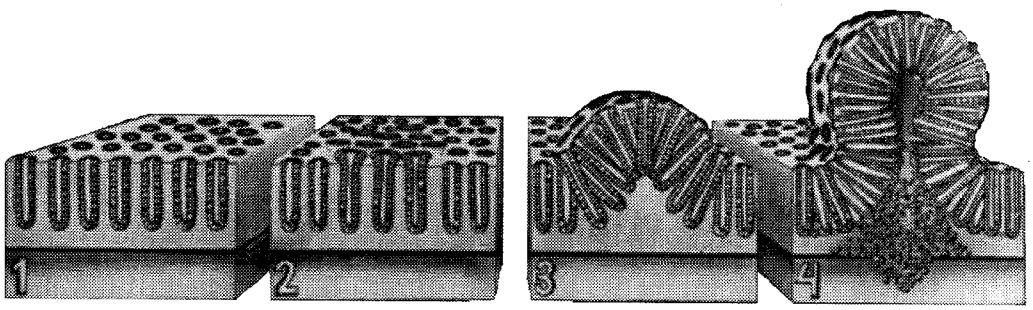

Figure 2. The suggested relation between normal crypts, aberrant crypts, ACF, adenomas and adenocarcinomas. 1: Normal crypts, 2: Aberrant crypt focus, 3: Adenoma, 4: Adenocarcinomas.

Bruce and colleagues (1993) have proposed two protocols, an initiation and a promotion protocol, for identifying factors likely to be carcinogenic to the colon as detected by the ACF assay.

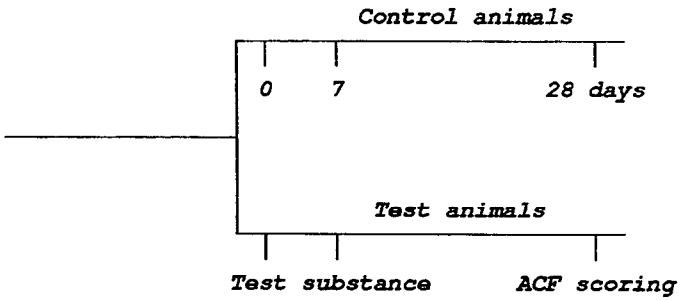

Figure 3. Protocol for initiators (adapted from Bruce et al. 1993).

In the protocol for initiators groups of mice or rats are given the test substance or the vehicle control/nothing at day 0 and 7 and ACF are scored in the colon at day 28.

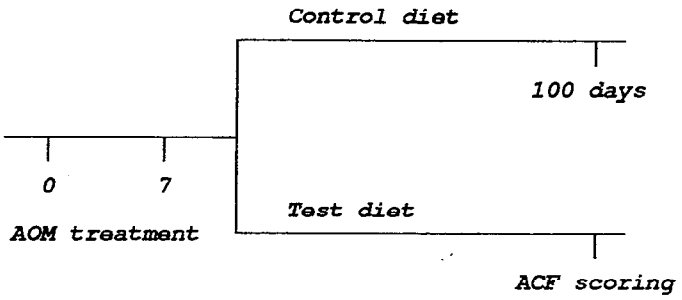

Figure 4. Protocol for promoters (adapted from Bruce et al. 1993).

In the promoter protocol the animals are initiated with AOM once a week for two weeks, and then treated with test diet or control diet. After 100 days the colons are scored for ACF. As seen in the ACF literature this protocol can be used to test possible inhibitors as well, and thus a more appropriate name would have been "Protocol for modulators".

Even though the protocols outlined by Bruce et al. (1993) have been used by several investigators, still it is very difficult to compare the results obtained by different investigators as a standardized protocol not yet has been adopted.

Other colon carcinogens than DMH-2HCl and AOM have been shown to induce ACF in rodent colon (APPENDIX, Table 1) and the lesions are clearly modulated by dietary factors, certain drugs, and miscellaneous other agents (APPENDIX, Table 2-4). Table 2, 3, and 4 give some examples of the modulating effect on the development of ACF of a series of compounds. The protocols used in the different experiments included in these tables are not comparable as several parameters which could influence the test results vary between the experiments, i.a. animal species and strain, colon carcinogen, dose and dosing regimen, the control diet used, duration of feeding with test diet, and feeding test diet in relation to time for carcinogen treatment. So, Tables 1-4 are meant only to give some examples of the different substances used for evaluating the ACF assay during the decade since the assay was described for the first time (Bird 1987).

That ACF are associated with colon carcinogenesis is supported by human data. Increased frequencies of ACF have been detected in normally appearing human colonic mucosa from patients with sporadic colon cancer and in patients with hereditary conditions that predispose to colon cancer, such as Gardner's syndrome (Pretlow et al. 1991) and familial adenomatous polyposis (FAP) (Roncucci et al. 1991). Many of the features seen in rodent ACF after microdissection and application of specific techniques have been detected in human ACF, i.e. dysplasia (Roncucci et al. 1991), proliferative activity (Roncucci et al. 1993), K-*ras* mutations (Pretlow et al. 1993, Smith et al. 1994, Jen et al. 1994, Yamashita et al. 1995), *APC* mutations (Smith et al. 1994, Jen et al. 1994) and over-expression of carcinoembryonic antigen (Pretlow et al. 1994b). Thus, the similarity of ACF in rodents and humans

lend support to the use of the rodent ACF assay as model for human colon cancer.

Most recently Moen et al. (1996) have emphasized the importance of taking genetic factors into account when evaluating the predictive value of the ACF assay for colon carcinogenesis. It has been known for long time that susceptibility to colon cancer is strongly strain dependent in mice, and partly strain dependent in rats, in which also males are more susceptible than females (Rogers & Nauss 1985a). CF1 and SWR mice are the most sensitive mouse strains to DMH-2HCl, reacting with almost 100% incidence of colon cancer (Thurnherr et al. 1973, Diwan et al. 1977), the C57BL/6J strain is moderately resistant to DMH-2HCl, producing a 43-48% tumour incidence (Diwan et al. 1977, Diwan & Blackman 1980), and AKR and DBA mice are extremely resistant to the carcinogen (Evans et al. 1974, Diwan et al. 1977). In rats Brown-Norway and Wistar-Furth are somewhat less susceptible than Fischer or Sprague-Dawley rats, which appear to be about equally susceptible (Rogers & Nauss 1985b).

This strain dependency has also been reported in the ACF assay. In mice strain differences have been found between CF_1 and C57BL/6J (Bird 1987, McLellan & Bird 1988a, 1988b, Stamp et al. 1993), between C57BL/6J and BALB/Cj mice (Kendall et al. 1992), between AKR/J, DBA/2J, SWR/J and P/J (Rosenberg & Liu 1995), and between BALB/Chhea, STS/A and CcS/Dem (9 strains) mice (Moen et al. 1996). Even though most of these studies suggested a relation between susceptibility to ACF and colon tumours, only the study by Moen et al. (1996) evaluated ACF and tumour outcome in the same animals under the same conditions. They found no agreement in three of the strains tested. Accordingly, the strain BALB/cHeA had high numbers of ACF and low numbers of tumours, indication that ACF not always progress to tumours; and the strains CcS-5 and STS/A had low numbers of ACF and high numbers of tumours. The authors suggest several mechanisms to this absence of an agreement between susceptibility to ACF and colon tumours and conclude that at least some genes that control the susceptibility to ACF are different from those involved in the susceptibility to colon tumours.

Likewise, strain differences in incidence of ACF have been detected between Fischer 344 and Lewis rats (Steffensen et al. 1995). The sex difference in colon carcinogenesis in rats has been demonstrated in ACF formation as well, by feeding Fischer 344 rats the heterocyclic amine PhIP. PhIP induces colon tumours in male, but not in female rats, and even though ACF were induced in both sexes, males had significantly more total ACF (x 3.5) than females (Ochiai et al. 1996).

The role of genetic susceptibility to colon carcinogenesis is also demonstrated by use of different intestinal mouse models. Mice with defects in the *adenomatous polyposis coli* (*APC*) gene are among the more promising in research on cancer of the digestive tract. Several mouse lineages heterozygous for specific mutations at the endogenous *Apc* gene have now been developed and characterized with respect to their intestinal tumour

multiplicity (Fodde et al. 1996). The mutation in the murine *Apc* gene render mice like *Min* (multiple intestinal neoplasia) (Moser et al. 1989), $Apc^{\Delta 716}$ (Oshima et al. 1995a,), and *Apc*1638N (Fodde et al. 1994, 1996), highly susceptible to spontaneous intestinal adenoma formation. ACF have been found to occur spontaneously in *Apc*1638N mice and at increased frequency after PhIP treatment (Sørensen et al. 1997). Increased number of ACF after treatment with PhIP has also been detected in C57BL/6J-*Min*/+ mice (Steffensen et al. 1996). Spontaneous occurrence of ACF was not seen in these C57BL/6J-*Min*/+ mice, but crypts with morphological features, different from what is found with AOM were detected. However, histopathological examination of these crypts showed dysplastic changes, similar to AOM-induced ACF (Paulsen et al. 1996a). In other studies spontaneous ACF were extremely rare in C57BL/6J*Min*/+ mice (Jacoby et al. 1996, Pierre et al. 1997), and in ongoing studies at the Institute of Food Safety and Toxicology with C57BL/6J*Min*/+ mice spontaneous occurrence of ACF has not been detected. $Apc^{\Delta 716}$ mice have been reported not to develop ACF spontaneously (Oshima et al. 1995b, 1997).

Additionally, the importance of genotype in the susceptibility for ACF formation has been proven by crossing male Min mice ($Apc^{+/-}$) with germ line DNA mitchmatch repair (*MMR*) gene (*Msh2*)-deficient female mice ($Msh2^{+/-}$), a mutation believed to be important during the rapid progression of the adenoma-to-carcinoma sequence in patients with hereditary nonpolyposis colorectal cancer. Spontaneous ACF were quite rare in $Apc^{+/-}/Msh2^{+/-}$ and $Apc^{+/-}/Msh2^{+/+}$ mice but were abundant in $Apc^{+/-}/Msh2^{-/-}$ (Reitmair et al. 1996a). Transgenic homozygous $Msh2^{-/-}$ mice also develop ACF spontaneously (Reitmair et al. 1996b). Tissues and cells having high levels of alkyltransferases are more resistant to mutagenic and carcinogenic effects of alkylating agents (Zaidi et al. 1995). When transgenic mice over-expressing *MGMT*, which codes for the human protein O^6-alkylguanine-DNA alkyltransferase, in their colon, and hence having increased alkyltransferase activity in the colon, were treated with AOM fewer ACF were developed compared with non-transgenic mice (Zaidi et al. 1995).

Most recently Paulsen et al. (1996b) and Feng et al. (1996) have found that the acetylator genotype in Syrian hamsters is a determinant of the development of ACF induced by the aromatic amine colon carcinogen 3,2'-dimethyl-4-aminobiphenyl (DMAB). *N*-acetylation capacity is linked to chemical-induced toxicity and human epidemiological studies are indicative of an association between colorectal cancer and rapid acetylator phenotype. After treatment with DMAB male Syrian hamsters of homozygous rapid acetylator genotype developed substantially more ACF than those of homozygous slow acetylator genotype.

In conclusion, the scientific literature reflects increasing evidence that the occurrence of ACF is linked to the induction of colon cancer both in animal models and in man. However, the experimental induction of ACF is influenced by several factors like e.g. species and strain of the laboratory animal, the diet, and the dose regimen.

OWN INVESTIGATIONS

In the first experiment archived colon tissue from a previous experiment (Thorup et al. 1992) using a classical rat model for carcinoma of the colon, was evaluated for the formation of ACF (Paper I). The rats had been treated with DMH-2HCl, and the modulating effect of a dietary fibre on colon cancer was investigated.

Due to the duration and cost of the classical rat colon cancer model the ACF assay was chosen as a possible assay in future studies. In the next studies the classical colon carcinogens, DMH-2HCl and AOM were used as inducers in rats and different diets were tested for their modulating effect on the development of ACF in colon (Paper II and III).

Most recently the heterocyclic amines PhIP and IQ, which are believed to be involved in the initiation of human colon cancer (Paper IV, V and VI) were used instead of the model colon carcinogens DMH-2HCl and AOM.

MATERIALS AND METHODS

A. Detection of ACF in colons from rats with carcinogen-induced tumours (Paper I).

Archived colon tissue from male Wistar rats in a one year study investigating the effect of a dietary fibre on experimentally induced colon cancer, was examined for the formation of ACF. In the one year study DMH-2HCl was used as initiator in a dosage of 20 mg/kg body weight, given by gavage once a week for 10 weeks. The dietary fibre tested was a beet fibre (Fibeta), and the two levels of Fibeta, added to the semisynthetic diet, corresponded to 0.7% and 14% dietary fibre in the rat diet. The study was designed to investigate the modulating effect of Fibeta during the periods of preinitiation, initiation, promotion, and progression. Groups of 30 rats, 60 controls, were initiated by DMH-2HCl, and throughout the study the rats were offered diets with different amounts of fibre on different time of the experimental period. As parts of the colon tissue from the rats in this study had already been processed for tumour characterization, only a minor part of the residual colon was selected for the ACF examination. The middle 1.5 cm of the major flexure (here defined as the colon segment situated from 7 cm to 12 cm from the junction between caecum and colon) was chosen. The ACF were grouped into small (1-3 crypts/focus), medium (4-6 crypts/focus), or large (≥7 crypts/focus) foci.

Experimental conditions, Paper I

Group	n	% Fibeta in Diet			DMH-HCl, mg/kg bw/wk
		8 Wks Preinit	10 Wks Init	30 Wks Postinit	
I	60	1	1	1	0
II	30	1	1	1	20
III	30	20	1	1	20
IV	30	20	20	1	20
V	30	20	20	20	20
VI	30	20	20	20	0
VII	30	1	20	1	20
VIII	30	1	1	20	20
IX	30	1	20	20	20

B. Modulating effect of different diets on the development of ACF and colon tumours in rats induced by DMH-2HCl or AOM (Paper II and III).

Two protocols were used to evaluate the modulating effect of different diets on carcinogen induced ACF.

The first protocol used oral administration of DMH-2HCl, 20 mg/kg body weight, once a week for 10 or 20 weeks to male Wistar rats. Three semisynthetic diets were tested: 1) a diet rich in sucrose and dextrin; 60% of the diet, 2) a high-fat-low-fibre diet (20% lard, 4% cellulose), and 3) a high-fat-high-fibre (20% lard, 20% beet fibre, 4% cellulose). The diets were fed during and after the DMH-2HCl dosing. ACF in the entire colon were recorded after 10 weeks (10 rats/ group), 20 weeks (8 rats/group), and 31 weeks (10 or 20 rats/group). The ACF were grouped into small (1-3 crypts/focus), medium (4-6 crypts/focus), large (7-9 crypts/focus), or extra large (≥ 10 crypts/focus) foci. The predictive value of the ACF was further evaluated by investigating whether there was a correlation with the incidence of colonic tumours after 31 weeks (10 or 20 rats/group).

Experimental conditions, Paper II[a,b]

Group	10 Wks DMH-2HCl	20 Wks DMH-2HCl	20 Wks DMH-2HCl + 11 Wks Post treatment
Control	10	8	10
Diet 1	10	8	10
Diet 2	10	8	20
Diet 3	10	8	20

a: Dimethylhydrazine dihydrochloride (DMH-2HCl), 20 mg/kg body wt/wk.
b: Values are expressed as no. of rats.

The second protocol used subcutaneous injections of AOM, 15 mg/kg body weight, once a week for two weeks to male rats. At the end of the dosing period the rats (14 rats/group) were given different test diets for 16 weeks. The 67% carbohydrate mix in the semisynthetic control diet was composed of 45% cornstarch, 45% potato starch, 5% sucrose, and 5% dextrin. In the three test diets the carbohydrate mix was replaced by 67% sucrose, 67% potato starch, and 67% cornstarch, respectively. The two starches tested were characterized by their high content of resistant starch (which physiologically may act as a dietary fibre), 55% in potato starch, or low content of resistant starch, 7% in cornstarch. At the end of the study all rat colons were evaluated for formation of ACF. The ACF were grouped into small (1-3 crypts/focus), medium (4-6 crypts/focus), large (7-9 crypts/focus), or extra large (≥ 10 crypts/focus) foci.

Experimental conditions, Paper III

Group	I	II	III	IV
Carbohydrate mix [a] %	67	0	0	0
Sucrose, %	0	67	0	0
Potato starch, %	0	0	67	0
Cornstarch, %	0	0	0	67

a: Carbohydrate mix: 45% cornstarch, 45% potato starch, 5% sucrose, 5% dextrin.

C. The heterocyclic amines 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) in the ACF assay (Paper IV, V and VI).

The food-related carcinogens selected for the next ACF studies were the two heterocyclic amines, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP).

In the first study (Paper IV) IQ and PhIP were tested in the ACF-assay in female C57BL/6J mice for 4 (4 mice/group) or 10 weeks (10 mice/group) using a feeding regimen with continuous low doses of IQ and PhIP to simulate human exposure. To evaluate the potency of IQ or PhIP as initiators in the ACF-assay, DMH-2HCl and AOM were tested as well.
Throughout the study groups of mice were offered a pellet diet, Altromin 1314, supplemented with 0.03% (wt/wt) IQ or 0.03% (wt/wt) PhIP. Additional groups were dosed intraperitoneally (10 ml/kg body weight) with AOM (5 mg/kg body weight) or DMH-2HCl (20 mg/kg body weight), respectively, once a week for two weeks. Four and ten weeks after initiation of the study mice were sacrificed and their colons evaluated for ACF formation.

Based on the results from the mouse study it was decided to use rats for the next study (Paper V). IQ was chosen as initiator and groups of male Fischer

rats (10/group) were given a semisynthetic pellet diet without or with 0.03% (wt/wt) IQ throughout the 10 weeks study. Two test diets were used: 1) a diet with a carbohydrate mix composed of 90% starches and 10% dextrin/sucrose and 2) a diet with 10% starches and 90% dextrin/sucrose. After 10 weeks, colons were examined for the formation of ACF.
The predictive value of the ACF was further validated by quantitating colonic tumours (data still under evaluation).

In the mouse study with IQ and PhIP (Paper IV), both agents were able to induce ACF in the colon of mice after respectively 4 and 10 weeks of exposure. In order to investigate the possible role of PhIP- and IQ-induced ACF in the development of colon cancer in mice, the third study on food mutagens as initiators of ACF was initiated (Paper VI). Colons from mice in other IQ- and PhIP-studies of much longer duration than that of paper IV were analyzed for ACF and the number of ACF at different time periods were evaluated. Wild type female mice, C57BL/6ByA and C57BL/6J, were used. The PhIP study included fifteen C57BL/6ByA mice given 0.03% (wt/wt) PhIP in the diet for 31 weeks, and with duration periods up to 78 weeks before ACF quantitation (Sørensen et al. 1996, Kristiansen et al. 1997). The IQ study included eighteen C57BL/6ByA mice given 0.03% (wt/wt) IQ in the diet for 24 weeks, and a study period of 40 weeks (Sørensen et al. 1996). The main purpose of these two studies was not to study ACF formation, and hence the studies were not designed as regular ACF-studies. However, due to the long duration of the studies, an investigation of the colons for ACF formation could add information about the fate of the early (4 and 10 weeks) recognized ACF.

Experimental conditions, Paper VI

Mouse strain	Treatment	Treatment period/Duration
C57BL/6ByA	± 0.03% PhIP, diet	31 Wks/31 or up to 78 Wks
C57BL/6ByA	± 0.03% IQ, diet	24 Wks/40 Wks
C57BL/6J	± 0.03% PhIP or IQ, diet	4 and 10 Wks

RESULTS

A. Detection of ACF in colons from rats with carcinogen-induced tumours (Paper I).

The results presented in this paper showed, in general, a statistically significant inverse relation between duration of intake of a high-fibre diet and number of rats with ACF, total number of ACF/rat and number of small

(1-3 crypt/focus) ACF/rat. The ACF results did not correlate with the tumour results, as no effect of the dietary fibre on colon tumour incidence in any stages of the carcinogenic process was seen (Thorup et al. 1992). Besides, no relation between high numbers of ACF and presence of colon tumours were seen in the individual rats.

B. Modulating effect of different diets on the development of ACF and colon tumours in rats induced by DMH-2HCl or AOM (Paper II and III).

The results in paper II showed that different dietary compositions were able to influence the development of ACF. Rats fed a diet high in refined carbohydrates had a statistically significant higher total number of ACF, and a higher number of small and medium ACF than the controls. The high-fat diet had no effect on the development of ACF, but adding dietary fibre to the high-fat diet resulted in a statistically significant reduction in the total number of ACF and the number of small and medium ACF. The effect of diets on development of ACF was not reflected in the tumour outcome (benign or malignant), as no statistically significant differences were seen between the groups. However, the rats on diet high in refined carbohydrate had the highest tumour incidence and the individual tumours were larger, reddish and cauliflower-like. Based on individual rat findings no relation between high numbers of ACF and presence of colon tumours was seen.

Study III revealed a marked inhibitory effect of potato starch on the development of ACF in the rats. Animals fed potato starch diet had a statistically significant lower number of total ACF as well as ACF in all categories but small. No effect of sucrose or cornstarch was seen. The animals receiving the potato starch diet showed a statistically significant reduced body weight gain after 5 weeks on experimental diet and a concomitant decrease in food consumption.

C. The heterocyclic amines 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) in the ACF assay (Paper IV, V, and VI).

In the study where mice were continuously fed low doses (0.03%) of IQ or PhIP (Paper IV), ACF were detected after 4 weeks. No ACF were seen in the AOM and DMH-2HCl dosed mice at that time. After 10 weeks ACF were found in all dosed groups. IQ-dosed mice had a statistically significant higher number of small and total ACF than the other groups after 4 as well as 10 weeks. Compared to the ACF induced by IQ or PhIP, AOM and DMH-2HCl induced a higher percentage of medium and large size ACF.

A comparable feeding regimen of continuous low IQ-doses (0.03%) throughout a study period of 10 weeks significantly increased the number of

small ACF as well as the total number of ACF in the colon of male F344 rats (Paper V). Additionally, the study showed that the incidence of IQ-induced ACF could be influenced by a diet high in refined carbohydrate. The total number and the number of small ACF were statistically significantly higher in rats given the high carbohydrate diet compared to rats fed the control diet. Whether the effect of the high carbohydrate diet on ACF induction is reflected in colon tumour incidence will be seen at termination of the study (data still under evaluation).

The results in paper VI, where colons from mice from IQ- and PhIP-feeding studies of different duration were investigated, showed that the number of ACF increased statistically significantly over time, and that the small ACF were predominant at all time-points. IQ induced ACF at a much higher number than PhIP, but no colon tumours were found in either IQ or PhIP dosed mice.

DISCUSSION

The studies of the present thesis have shown that ACF are induced in rodent colon by colon carcinogens such as DMH-2HCl, AOM, IQ, and PhIP and that the incidence of induced ACF can be modulated by different dietary components.

The best way to evaluate the tumour predictive value of the assay is to assess the ACF and tumour incidence in the same animals under the same conditions.

Both of the studies proceeding to tumour formation (Paper I and II) did not show a clear relation between the outcome of ACF and tumours with any of the dietary components tested. Neither a clear relation, based on individual rat findings, between high numbers of ACF and presence of colon tumours was seen. A third study is still under evaluation.
In general, the most consistent finding in all experiments was that if a modulating effect of a tested diet was found, the effect was exerted on the total number of ACF and the number of small (1-3 crypt/focus) ACF. In one example using the protocol proposed by Bruce et al. (1993) (AOM injection, once a week for 2 weeks) a modulating effect of a high potato starch diet was seen in all categories of ACF but small (Paper III).

Accumulating evidence now supports the hypothesis that total numbers of ACF and numbers of small ACF are not significantly related to tumour incidence. Pretlow et al. (1992a), Magnuson et al. (1993), Zhang et al. (1992), Bird (1995b), Shivapurkar et al. (1994, 1996) all found that the number of ACF with multiple crypts rather than the total number of ACF

correlated with tumour incidence. In the light of this hypothesis our results could be interpreted as if all the tested diets except one had little effect on the endpoint colon cancer. However, either the choice of different protocols could explain the difficulties in interpreting the results, or the ACF and colon tumours could represent two parallel independent events as a consequence of the cancer induction, i.e. the ACF not being preneoplastic per se.

Study design

Compared to the protocol for ACF studies proposed by Bruce et al. (1993) we used multiple DMH-2HCl doses (20 mg/kg body weight for 10 or 20 weeks) in paper I and II. We decided to use repeated doses of carcinogen due to difficulties in obtaining colon tumour incidences high enough to enable the measuring of an inhibitory effect of a test compound. Thus, in the experiment (Thorup et al. 1992) that formed the basis for paper I, using DMH-2HCl, 20 mg/kg body weight once a week for 10 weeks, only approximately 30% of the rats developed colon tumours. With the aim of obtaining a higher incidence of colon tumours a study (unpublished) was initiated using doses of 40.5 mg/kg body weight or 81 mg/kg body weight DMH-2HCl, once a week for 10 weeks. This study showed that even though it was possible to induce colon tumours at an incidence of about 80%, serious toxic effects to vital organs such as liver and kidney as well as tumours of the kidneys and Zymbal gland and deaths were induced. A relatively low incidence of colon tumours as obtained in our studies with Wistar rats by a dosing regimen normally capable of inducing a substantially higher amount of colon tumours (Shamsuddin 1983), has also been observed in studies with Wistar rats conducted at the TNO-CIVO Toxicology and Nutrition Institute Zeist, The Netherlands (C.F. Kuper, personal communication).

Our use of multiple doses of the carcinogen, has been questioned by Bird (1995a). Yet little knowledge is available about the effect of one vs. multiple injections of a colon carcinogen on the induction and growth features of ACF. By injecting Sprague Dawley rats subcutaneously with one, two or four injections of AOM (10 mg/kg/week) followed by sacrifice at week 6, 14 and 28, Bird (1995a) found that: "the initial response to repeated exposure to AOM was characterized by a lag period during which ACF appeared indolent before the growth stimulating effect of AOM became evident". Therefore it was suggested that enumeration of the number and growth characteristics of ACF determined immediately after repeated treatment with a colon carcinogen may not predict tumour incidence.

How critical the carcinogen dose is in ACF induction was recently demonstrated by Bird & Lafave (1995). ACF induced by a single dose of 5 mg/kg AOM and scored after 8 weeks consisted of crypts that were larger in size and responded differently to growth modulation by diet than those induced by 20 mg/kg AOM. The authors proposed that the marked

cytotoxicity known to be induced by the high dose of AOM, and followed by hypo- then hyperproliferative responses, results in more stable preneoplastic lesions as a result of their clonal selection and expansion. A low dose of AOM would then be expected to have less effect (Bird & Lafave 1995).

Our evaluation of only a small segment of the colon (Paper I) has been criticized (Bird 1995a) for not having been validated, and only used by one other research group (Hardman et al. 1991). Nevertheless similar effect of the same dietary fibre, i.e. no association between ACF and tumour incidence, was found in the next study evaluating the entire colon (Paper II). Even though the reservation expressed by Bird (1995a) seems relevant, it is possible to find other examples in the literature on the ACF assay where the entire colon has not been evaluated (Duranton et al. 1997, McLellan et al. 1991a, Pereira et al. 1996a, Zarkovic et al. 1993, Young et al. 1996). In addition, one must bear in mind, that all the human data, which are also used in support of the hypothesis that ACF are putative precursor lesions to colon cancer, have been obtained from only minor segments of the human colon.

Rodent species and strain/stock

Even though Wistar rats are reported to be less susceptible to colon cancer than Fischer rats (Rogers & Nauss 1985b), no difference with respect to tumour incidence was found in our study (unpublished) comparing Sprague-Dawley, Fischer 344, and Wistar male rats, treated with DMH-2HCl, 20 mg/kg body weight/week for 10 weeks. Therefore, Wistar rats were chosen in most of the studies presented here, as this strain has been used in toxicologic research at the Institute for more than 20 years, and thus a considerable amount of experience, biological as well as pathological, has accumulated.

Even though the ACF literature comprises many studies using mice, the use of mice has been questioned. In a study by Carter et al. (1994) using DMH-2HCl-treated mice the authors proposed that ACF have little if any malignant potential in the mouse. Likewise, Steele & Kelloff (1993) testing 42 chemopreventive agents found that all of the false positive data (positive crypt/negative tumour) were derived from mouse tumour studies and the authors indicated that some species differences may exist. Additionally, no obvious relationship between susceptibility to ACF and susceptibility to colon adenomas has been found in certain mouse strains, suggesting that some genes that control the susceptibility to ACF are different from the genes that are involved in the susceptibility to colon adenomas (Moen et al. 1996).

Dietary fibre

A significant effect of a dietary fibre (a beet fibre) on the development of ACF without a concomitant effect on tumour incidence was seen in both tumour studies (Paper I and II). In both studies a statistically significant reduction was seen in the total number of ACF and the number of small ACF. If the hypothesis that a reduction in the total number of ACF and the number of small ACF is not predictive for a reduction in tumour incidence is true, then the study did not show that the dietary fibre tested had a protective effect on the induction of colon cancer in rats with the protocol used in the two studies.

A reduction in ACF after feeding dietary fibre to carcinogen treated rats has been shown with flaxseed (total no. of ACF) (Serraino & Thompson 1992, Jenab & Thompson 1996), wheat bran (total no. of ACF) (Alabaster et al. 1995), (total no. and ACF≥4 crypts) (Ferguson & Harris 1996), (total no. of ACF) (Ishizuka & Kasai 1996) and low risk diets (high fibre and calcium, low fat) (total no. and medium size ACF) (Hardman et al. 1991). Bearing the above hypothesis in mind, the stated protective effect on development of colon cancer of the different fibres in these experiments could be questioned.

Fat

The data in paper II did not support a modulating effect of a high-fat diet on the development of ACF shown in other studies (Shivapurkar et al. 1992, McLellan & Bird 1988b, Bird & Lafave 1995, Bird et al. 1996, Morotomi et al. 1997). Neither did the high-fat diet have any effect on tumour incidence.

Refined carbohydrates

The enhancing effect of diets high in refined sugars on ACF development was demonstrated in two of the three studies with high sucrose diets. The modulating effect on ACF by refined carbohydrates seemingly was inducer dependent.

An enhancing effect was seen after ACF-induction with DMH-2HCl (20 mg/kg weekly). The total number of ACF increased 2.7-fold after 10 weeks feeding compared to controls. The highest increase was seen in the number of small ACF, but the number of medium, large and extra-large ACF were increased as well (Paper II).

Rats given continuous low doses of IQ (0.03%) for 10 weeks together with a diet rich in refined carbohydrates had a statistically significantly higher number of small and total number of ACF compared to IQ-dosed animals given standard diet. The increase in total number was approximately two

fold. The highly refined carbohydrate diet induced a lower, but statistically not significant number of medium size ACF (Paper V).

When AOM was used as initiator (15 mg/kg weekly for two weeks) no effect was seen on the total number of ACF after 10 weeks feeding on a high carbohydrate diet. The number of medium, large and extra-large ACF were all reduced compared to the controls and only the number of small ACF was increased (1.4-fold) (Paper III). Inducer dependency is further discussed for other compounds on page 31.

The enhancing effect of diets high in refined carbohydrates on development of ACF has been reported by others (46 % sucrose in the diet, increase in ACF with 3-6 AC/foci, Caderni et al. 1991); (10 g/kg body weight as one bolus, increase in total no. of ACF, Stamp et al. 1993), and epidemiological studies have indicated that refined sugar may be a risk factor in human colo-rectal cancer (Bristol et al. 1985, Macquart-Moulin et al. 1987, Tuyns et al. 1987, 1988, La Vecchia et al. 1993).

Starch

Diets containing relatively large amounts of resistant starch, seemed to have a protective role on the development of ACF (Paper II and III).

In paper II the carbohydrate pool in the control diet mainly consisted of starches (45% cornstarch, 45% potato starch, 5% sucrose, and 5% dextrin), and compared with the high sucrose diet group (45% sucrose, 45% dextrin, 5% potato starch, and 5% cornstarch) fewer ACF were seen in the control group. Both potato starch and cornstarch contain resistant starch (55% and 7%, respectively), which physiologically may act as a dietary fibre, and hence explain the difference in ACF between the two groups.

As resistant starch may be of importance as a protective factor also in human colo-rectal cancer (Cummings & Bingham 1987, Bingham 1990, Cassidy et al. 1994), diets with different amounts of resistant starch were tested in the next study (Paper III). Potato starch (67% of the diet ≈ 37% resistant starch) had a marked effect on ACF development, as a statistically significant lower number in all categories of ACF but small was seen compared to controls (30 % potato starch ≈ 17% resistant starch in the diet). An effect of the lower daily caloric intake observed in this group can not be excluded. Lasko & Bird (1994) have shown that caloric restriction reduces the total number of AOM-induced ACF and colonic adenomas in rats. The inhibitory effect of caloric restriction on tumour development has been known for decades.

The opposite effect, i.e. an enhancing effect of potato starch on carcinogen induced ACF (95% being of small size) has recently been reported in Sprague Dawley rats, an effect which was also reflected in the tumour incidence (Young et al. 1996). No effect on body weight was seen in the potato

starch group in this study. In the potato starch diet 20% of the carbohydrate mix (72% of the diet) was raw potato starch ≈ 14.4% potato starch in the test diet. The resistant starch content in the diet was reported as 20 % wt/wt of total starch based on resistant starch analyses of the pellets ≈ 14.4% resistant starch in the diet. The animals in the Young et al. (1996) study were fed this diet for 31 weeks, and were injected 10 times subcutaneously with DMH-2HCl, 20 mg/kg body weight/week from week 2 to 11. The use of 10 weeks carcinogen treatment, simultaneously with feeding the test diet compared to the two weeks treatment prior to the test diet feeding in paper III in combination with a potato starch content in the diet approximately 2.5 times lower than that in paper III, and finally a longer experimental period than ours (31 vs. 16 weeks) all are factors which could explain the different results obtained. In other rat studies, resistant starch added to the diet has been found to decrease the number of ACF (Mazière et al. 1996 (primarily small size), Perrin et al. 1996).

The diet which was high in cornstarch (67% of the diet ≈ 5% resistant starch) had no inhibitory effect on the formation of ACF. The amount of resistant starch in the cornstarch diet was somewhat lower than in the control diet, 5% vs. 17%, which might explain the higher, although statistically not significant, number of total and small ACF found in this group compared to controls. Cornstarch diets (46 % of the diet) have earlier been described as having a reducing effect on the size of the DMH-2HCl-induced ACF (Caderni et al. 1991).

Colon cancer inducers of dietary relevance

The carcinogenicity of the most widely used carcinogens for studying colon carcinogenesis in rodents, DMH-2HCl and AOM, is not definitively known for humans. For the identification of the component of the human diets that might be responsible for the causes of colorectal cancer the use of food-related carcinogens in the ACF assay might in a better way reflect the diet-related carcinogenesis in humans.

The food-related carcinogens selected for the ACF studies were the two heterocyclic amines, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP).

Mouse studies

In the mouse study (Paper IV), feeding continuous low doses (0.03%) of IQ or PhIP, ACF (almost solely small ACF) were detected after both 4 and 10 weeks. Seemingly, IQ is a much more potent ACF inducer in the mouse colon than both PhIP, DMH-2HCl and AOM with respect to total number of ACF and small ACF. The well known mouse colon carcinogens AOM and DMH-2HCl induced a substantially higher percentage of medium or large size ACF than PhIP or IQ supporting the theory that ACF with a higher crypt multiplicity is more predictive concerning the development of

colon cancer (Pretlow et al. 1992a, Magnuson et al. 1993, Zhang et al. 1992, Bird 1995b, Shivapurkar et al. 1994, 1996).

IQ and PhIP have earlier been reported to induce ACF in female CD1 mice in a three week study by Tudek et al. (1989) in which a few ACF were observed after administration of two doses of IQ (total 400 mg/kg body weight) and PhIP (total 150 mg/kg body weight). As neither IQ nor PhIP target colon in mouse carcinogenicity studies performed on the hybrid mouse CDF1 [(BALB/Cann x DBA/2N)F_1] (Ohgaki et al. 1984a, Esumi et al. 1989), ACF induced by IQ and PhIP in mice probably do not represent an early stage of colon cancer. An explanation might be that PhIP and IQ are only weakly carcinogenic to the colon compared to other organs, and therefore tumours arise in the liver, forestomach and lung in the case of IQ (Ohgaki et al. 1984a), and in the lymphoid tissue in the case of PhIP (Esumi et al. 1989) long before they arise in the colon. Whether long-time feeding with PhIP induces tumours in other organs incl. colon in mice is yet not known as only the data on lymphomas have been published, and a thorough histological examination of this carcinogenicity study still remains to be reported (Esumi et al. 1989).

In order to expand the knowledge about possible histopathological changes due to longer treatment time with PhIP, some of the mice from a study recently performed at the Institute (Sørensen et al. 1996) where mice were fed diets with 0.03% PhIP for seven months, were maintained for another 11 months on control diet without PhIP (Kristiansen et al. 1997). This study confirmed that PhIP is a potent mouse lymphomagen and additionally showed that treatment with 0.03% in the diet for the first seven months of a life time study (18 month) gave rise to very few and sporadic tumours in other tissues, but none in colon, indicating weak if any carcinogenicity of PhIP to organs other than the lymphatic system under the study design used (Kristiansen et al. 1997).

As IQ and PhIP have not been found to have colon as target organ in carcinogenicity studies but nevertheless induced ACF in colon of mice, the possible role of PhIP- and IQ-induced ACF in the development of colon cancer in mice was investigated by analyzing colons from mice from other IQ- and PhIP-studies of much longer duration for ACF (Paper VI). The results of these studies showed that the total number of ACF increased statistically significantly over time, and that the small ACF were predominant (95-100%) at all time-points. IQ induced ACF in a much higher number than PhIP, but no colon tumours were found either in IQ or PhIP dosed mice. As the model mouse colon carcinogens DMH and AOM have been shown to induce ACF with relatively high crypt multiplicity (Koratkar & Rao 1997, Paper IV) compared to the low crypt multiplicity (1-3 AC/Focus) found after IQ- or PhIP-treatment, this study could supports the hypothesis that the occurrence of ACF with low crypt multiplicity is probably not predictive for tumour outcome.

Most recently Okonogi et al. (1997) have found that two other heterocyclic amines, 2-amino-9*H*-pyrido[2,3*b*]indole (AαC) and 2-amino-3,8-dimethyl-

imidazo[4,5-*f*]quinoxaline (MeIQx), were able to induce ACF in C57BL/6N mice and most of the foci consisted of one or two aberrant crypts. As AαC and MeIQx not have been found to induce colon tumours in carcinogenicity studies in CDF1 mice (Ohgaki et al. 1984b, 1987) the authors suggest that the large intestinal carcinogenicity of heterocyclic amines in mice needs to be re-examined using susceptible strains, such as C57BL/6N. However, our own examination of PhIP in C57BL/6J mice fed 0.03% in the diet for the first 7 months of a life time study (Kristiansen et al. 1997) failed to induce colon tumours although ACF earlier had been detected with PhIP in this mouse strain (Paper IV).

The conclusion drawn from our mouse studies was, that if cooked food mutagens as IQ or PhIP are to be used as initiators in the ACF test, the use of rats might be a better choice since: 1) in carcinogenicity studies with rats, colon is target organ for both IQ (Takayama et al. 1984a) and PhIP (Ito et al. 1991), 2) in rats induction of ACF in the colon has been demonstrated after both IQ (Tudek et al. 1989, Liew et al. 1995, Tachino et al. 1995, Ferguson & Harris 1996, Xu et al. 1996) and PhIP (Tudek et al. 1989, Takahashi et al. 1991, Hasegawa et al. 1993, Weisburger et al. 1994, Guo et al. 1995a, Ochiai et al. 1996a, 1996b), and 3) the distribution of ACF in the colon of IQ or PhIP dosed rats was comparable with that in rats given AOM or DMH-2HCl and correlated well with the distribution of tumours (Tudek et al. 1989, Hasegawa et al. 1993).

Rat studies

As IQ had been shown to induce ACF in the rat colon (Tudek et al. 1989, Hasegawa et al. 1993), but yet not had been used for evaluating the modulating effects of dietary components a rat study was initiated. With a feeding regimen of continuous low IQ-doses (0.03%) throughout a study period of 10 weeks a significant effect on the induction of the number of small ACF as well as total number of ACF was seen (Paper V). Additionally, it was shown that the incidence of IQ-induced ACF could be modulated by a diet high in refined carbohydrate. However, the fact that IQ is a rat colon carcinogen, together with the demonstrated induction of small and total number of ACF when scoring after 10 weeks, does not support the hypothesis that the presence of a high number of ACF with high crypt multiplicity is predictive for tumour outcome. Whether the effect of IQ and of the high carbohydrate diet on ACF induction is reflected in colon tumour incidence will be seen at termination of the study (data still under evaluation).

None of the studies with IQ reported until now (Tudek et al. 1989, Liew et al. 1995, Tachino et al. 1995, Ferguson & Harris 1996, Xu et al. 1996) have examined the ACF and tumour incidence in the same experiment under the same conditions. Only the studies by Ferguson & Harris (1996) and Tachino et al. (1995) have grouped the ACF data according to size of individual crypts constituting each ACF. In both studies a substantial percentage of the IQ induced ACF contained 4 AC/focus (23% of total ACF, Tachino et al. 1995) or >4 AC/focus (26% of total ACF, Ferguson & Harris 1996)

supporting the hypothesis of ACF ≥ 4 AC/focus being predictive for colon cancer. A possible explanation to the different results obtained by Tachino et al. 1995, Ferguson & Harris 1996 and paper V, could be that paper V used a continuous low dose (0.03% (wt/wt) in feed) of IQ for 10 weeks (a total dose of approximately 210 mg/kg), compared to Tachino et al. (1995) which used 130 mg/kg body weight by oral gavage on alternating days for 2 weeks (a total dose of approximately 910 mg/kg) with ACF scoring 12 weeks after the initial carcinogen treatment, and Ferguson & Harris (1996) which used three doses of IQ (50 mg/kg body weight) at weekly intervals (a total dose of approximately 150 mg/kg) and scoring 10 weeks after the first IQ treatment.

Choice of initiator

The choice of compound for induction of ACF and the possible effect on the modulating effect of test compound is illustrated by various experiments.

Chlorophyllin (CHL) is an inhibitor of PhIP-induced ACF (Guo et al. 1995a) and IQ-induced rat colon cancer (Guo et al. 1995b). However, CHL neither decreases nor increases AOM-induced ACF (Pereira et al. 1994), and when DMH-2HCl is used as initiator CHL acts as a promoter of colon cancer (Nelson 1992). Similarly, indole-3-carbinol (I3C) is an inhibitor of PhIP-induced (Guo et al. 1995a) and IQ-induced ACF (Xu et al. 1996), but enhances DMH-2HCl-induced colon carcinogenesis (Pence et al. 1986).

CONCLUSION

In conclusion, the studies included in the present thesis have shown that ACF are induced in rodent colon by colon carcinogens such as DMH-2HCl, AOM, IQ, and PhIP and that the incidence of induced ACF can be modulated by different dietary components.

Most of the current data from the ACF literature, and to some extent from the studies presented, support the evidence that some parameter(s) of the ACF assay do predict tumour incidence. Most of this evidence has been indirect:

- ACF are induced specifically by colon carcinogens in rodents;
- substances that promote colon cancer promote the development of ACF;
- substances that inhibit development of colon cancer inhibits ACF formation as well;
- genetic changes observed in colon tumours are also found in ACF;
- ACF are found in the human colon in patients at increased risk of colon cancer.

As no clear relation between the outcome of ACF and tumours was found in the present studies, the results could indicate that ACF and colon tumours represent two parallel independent events as a consequence of the cancer induction, i.e. the ACF not being preneoplastic per se. Or, the results could propose that only some of the ACF are precursor lesions for colon tumours and that the time point in the colon carcinogenic process, at which the ACF are scored is not the optimal for scoring a true relation between ACF and colon tumours. Finally the bare recording of the number of ACF and their crypt multiplicity is possible not sufficient to characterize the predictive value of ACF for tumour induction.

To determine which is the best parameter(s) of the ACF assay in identifying the ACF at risk is not yet possible. The use of total number of ACF as parameter is not sufficient. More likely it is the number of ACF with multiple crypts that correlates with tumour incidence. Additional studies are needed to determinate the optimal parameter(s) of this assay and to further validate the ACF assay with test for tumour incidence, before ACF can be used as reliable intermediate biomarkers, and subsequently being models for human colon cancer.

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APPENDIX

TABLE 1. EFFECT OF DIFFERENT CARCINOGENS ON COLON IN RODENTS

CARCINOGEN		COLON CARCINOGEN	ACF	References
DMH-2HCl	1,2-dimethylhydrazine dihydrochloride	x	x	Druckrey et al. 1967, McLellan & Bird 1988a
AOM	azoxymethane	x	x	Ward et al. 1973, Bird 1987
MNU	N-nitroso-N-methylurea	x	x	Narisawa et al. 1976, McLellan & Bird 1988a
MNNG	N-methyl N'-nitro-N-nitrosoguanidine	x	x	Reddy et al. 1975, Bilbin et al. 1992
MCA	3-methylcholanthrene	x	x	McLellan & Bird 1988a
PhIP	2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine	x	x	Ito et al. 1991, Takahashi et al. 1991
IQ	2-amino-3-methyl-imidazo[4,5-*f*]quinoline	x	x	Takayama et al. 1984a, Tudek et al. 1989
Glu-P-1	2-amino-6-methyldipyri-dol[1,2α:3',2'-*d*])imidazole	x	x	Takayama et al. 1984b, Tudek et al. 1989
MeIQ	2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline	x	x	Kato et al. 1989, Tudek et al. 1989
AFB1	Aflatoxin B_1	x	x	Newberne & Rogers 1973, Tudek et al. 1989
DMAB	3,2'-dimethyl-4-aminobiphenyl	x	x/0	Walpole et al. 1952, McLellan & Bird 1988a, Steffensen et al. 1995
Hydrazine sulphate		-	..	McLellan & Bird 1988a
2-aminobiphenyl		..	..	McLellan & Bird 1988a
Benzo(a)pyrene		-	x/..	McLellan & Bird 1988a, Zarkovic et al. 1995
Methylurea		-	..	McLellan & Bird 1988a
Colchicine		-	..	McLellan & Bird 1988a

TABLE 2. EFFECT OF DIFFERENT PLANT CONSTITUENTS AND THEIR DERIVATIVES ON COLON CARCINOGEN-INDUCED TUMOURS AND ACF IN RODENT COLONS

COMPOUND	INHIBITION OF:		PROMOTION OF:		
	COLON CANCER	ACF	COLON CANCER	ACF	References
PCA, protocatechuic acid	x	x			Kawamori et al. 1994, Tanaka et al. 1993
N-acetyl-L-cysteine	x	x/0			Wilpart et al. 1986, Pereira & Khoury 1991, Pereira et al. 1994
Diallyl sulphide	x	x/0			Wargovich 1987, Wargovich et al. 1992a, Pereira & Khoury 1991
S-allyl cysteine	x	x			Sumiyoshi & Wargovich 1990, Hatono et al. 1996
Ellagic acid	0	0			Pereira & Khoury 1991, Rao et al. 1991
Phenethyl isothiocyanate	?	0			Pereira & Khoury 1991
Genistein	?	x			Barnes et al. 1993
Rutin	x	x/0			Deschner et al. 1991, Pereira et al. 1994, Wargovich et al. 1996
Fumaric acid	?	x			Barnes et al. 1993
Dimethylfumarate	0/x	x			Barnes et al. 1993, Rao et al. 1995
Curcumin, diferuloylmethane	x	x/0			Wargovich et al. 1992a, Huang et al. 1993, Rao et al. 1993a, Pereira et al. 1996b
Quercetin		0	x		Pereira et al. 1996b
Chlorophyllin	x	x/0	x		Guo et al. 1995a, 1995b, Nelson 1992, Pereira et al. 1994
18-beta-glycyrrhetinic acid	0	x			Reddy et al. 1992, Wargovich et al. 1992b
Indole-3-carbinol	?	x	x		Guo et al. 1995a, Pence et al. 1986, Xu et al. 1996
Astaxanthin	x	x			Tanaka et al. 1995
Canthaxanthin	x	x			Tanaka et al. 1995
Caffeic acid esters	?	x			Rao et al. 1993b
d-limonene	?	x			Kawamori et al. 1996

TABLE 2. cont. EFFECT OF DIFFERENT PLANT CONSTITUENTS AND THEIR DERIVATIVES ON COLON CARCINOGEN-INDUCED TUMOURS AND ACF IN RODENT COLONS

COMPOUND	INHIBITION OF:		PROMOTION OF:		
	COLON CANCER	ACF	COLON CANCER	ACF	References
Phytate	x	x			Shamsuddin et al. 1988, Pretlow et al. 1992a
Wheat bran	x	x			Greenwald & Lanza 1986, Alabaster et al. 1995, Ferguson & Harris 1996
Flaxseed	?	x			Serraino & Thompson 1992, Jenab & Thompson 1996
Beet root	0	x			Thorup et al. 1994 (Paper I)
Corn starch	?	x/0			Caderni et al. 1991, Thorup et al. 1995 (Paper III)
Potato starch	?	x	x	x	Thorup et al. 1995 (Paper III), Young et al. 1996
Sucrose			?	x/0	Stamp et al. 1993, Thorup et al. 1995 (Paper III)
Fructose			?	x	Stamp et al. 1993
Vitamin E	x/0	x	x		Toth & Patil 1983, Cook & McNamara 1980, Shivapurkar et al. 1995
Beta-carotene	x	x			Alabaster et al. 1995, Komaki et al. 1996
Ascorbyl palmitate	x	x/0			Wargovich et al. 1992a, 1996, Rao et al. 1995
Selenium	x	x			Jacobs et al. 1981, Reddy et al. 1994
Perilla oil	x	x			Narisawa et al. 1994, Onogi et al. 1996, Komaki et al. 1996
S-methyl methane thiosulphate	x	x			Kawamori et al. 1995a

TABLE 3. EFFECT OF DIFFERENT DRUGS ON COLON CARCINOGEN-INDUCED TUMOURS AND ACF IN RODENT COLONS

COMPOUND	INHIBITION OF:		PROMOTION OF:		
	COLON CANCER	ACF	COLON CANCER	ACF	References
Disulfiram	x	x			Wattenberg 1975, McLellan & Bird 1991
Aspirin	x	x/0			Craven & De Rubertis 1992, Pereira et al. 1994, Mereto et al. 1994
DFMO, Alpha-difluoromethylornitine	x	x			Rozhin et al. 1984, Pereira & Khoury 1991, Rao et al. 1991
Ibuprofen	x	x/0			Bandaru et al. 1992, Pereira et al. 1994, Wargovich et al. 1996
Piroxicam	x	x			Nigro et al. 1986, Rao et al. 1991, Pereira et al. 1994, 1996a
Sulindac	x	x			Moorghen et al. 1988, Barnes et al. 1993
Indomethacin	x	x			Charalambous et al. 1996, Narisawa et al. 1981
Suramin	?	x			Pereira et al. 1994
Ketoprofen	x	x			Reddy et al. 1992, Wargovich et al. 1992a
Nimesulide	?	x			Takahashi et al. 1996
Triazine derivatives	?	x			Hirose et al. 1996
KYN-5, 5-hydroxy-4-(2-phenyl (*E*)ethenyl)-2(5H)-furanone	x	x			Kawamori et al. 1995b
22-oxa-calcitrol	x	x			Otoshi et al. 1995
Oltipraz	x	x			Wargovich et al. 1992a, Rao et al. 1993c

TABLE 4. EFFECT OF MISCELLANEOUS COMPOUNDS AND TREATMENTS ON COLON CARCINOGEN-INDUCED TUMOURS AND ACF IN RODENT COLONS

COMPOUND	INHIBITION OF:		PROMOTION OF:		
	COLON CANCER	ACF	COLON CANCER	ACF	References
BHA, 3-tert-butyl-4-hydroxyanisol	x	x			Wattenberg & Sparnins 1979, Lam & Zhang 1991
PPITC, phenylpropyl isothiocyanat	?	x			Lam & Zhang 1991
Calcium salts	x/0	x/0			Newmark et al. 1984, McSherry et al. 1989, Wargovich et al. 1990, Welberg et al. 1991, Barnes et al. 1993, Pereira et al. 1994
Caloric restriction	x	x			Reddy et al. 1987, Lasko & Bird 1995
Insulin			x	x	Tran et al. 1996, Corpet et al. 1997
Cholesterol			x	x	Klurfeld et al. 1983, Kendall et al. 1992, El-Sohemy et al. 1996
Beef tallow / lard			x/0	x/0	Reddy et al. 1977b, McLellan & Bird 1988b, Kristiansen et al. 1995 (Paper II), Bird et al. 1996
Conjugated linoleic acid	?	x			Liew et al. 1995
DHA, docohexaenoic acid (fish oil)	x	x			Takahashi et al. 1994
Bifidobacteria	x	x			Koo & Rao 1991, Reddy & Rivenson 1993
BT, 2-n-butylthiophene	x	x			Lam & Zhang 1991, 1993
OT, 2-n-octylthiophene	?	x			Lam & Zhang 1991
HF, 2-n-heptylfuran			0	x	Lam & Zhang 1993
Sphingomyeline	x	x			Dillehay et al. 1994
Dehydroepiandrosterone	x	x		x	Nyce et al. 1984, Pereira & Khoury 1991, Wargovich et al. 1996
Alpha-difluoromethylornithine	x	x			Nigro et al. 1986, Pereira & Khoury 1991
Cholic acid		x	x	x	Cohen et al. 1980, Bird 1991, Seraj et al. 1997
Chenodeoxycholic acid			x	x	Reddy et al 1977a, Sutherland & Bird 1994

OWN REFERENCES

PAPER I

Influence of a Dietary Fiber on Development of Dimethylhydrazine-Induced Aberrant Crypt Foci and Colon Tumor Incidence in Wistar Rats

Inger Thorup, Otto Meyer, and Eva Kristiansen

Abstract

Formation of aberrant crypt foci (ACF) in archived colon tissue from animals in a previous study was examined. The animals were fed a semisynthetic casein-based diet in which the carbohydrate pool was substituted with a dietary beet fiber (Fibeta) as the only source of fiber. Oral doses of dimethylhydrazine dihydrochloride (DMH-2HCl, 20 mg/kg body wt) once a week for 10 weeks were used as initiator. The rats were fed different levels of the fiber in a preinitiation period, during initiation, or in a postinitiation period.

In general, the results showed a statistically significant inverse relation between duration of intake of high-fiber diet and number of animals with ACF, as well as the total number of ACF and number of small ACF (1–3 crypts) per affected animal. The previously reported data showed no protective effect of the dietary fiber at any stage of the colorectal carcinogenic process. The lack of correlation between the outcome of ACF and tumors could be related to the observation that statistically significant differences between groups were seen only in the total number of ACF and number of small ACF.

The hypothesis that ACF are preneoplastic lesions needs to be supported by further experimental data. The present state of knowledge could indicate that ACF represent true preneoplastic lesions progressing into colon tumors or that ACF and colon tumors represent two parallel independent events as a consequence of the cancer initiation (i.e., the ACF not being preneoplastic lesions per se).

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Introduction

A large number of investigations on the possible modifying effect of dietary fiber on experimentally induced colorectal cancer have been performed during the last decades.

In a recent experiment in our laboratory, the modifying effect of two levels of a well-specified dietary fiber, Fibeta (a beet fiber), during preinitiation, initiation, and postinitiation of colorectal cancer has been studied in rats initiated with dimethylhydrazine dihydrochloride (DMH-2HCl). A protective effect of the dietary fiber was not found at any stage of the colorectal carcinogenic process, whereas the continuous feeding with the high-fiber diet resulted in a statistically significant increase in volatile fatty acids, especially butyric acid, which is suggested to possess anticarcinogenic properties (1).

Aberrant crypt foci (ACF) in the colonic mucosa have been hypothesized to represent

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precursor lesions of chemically induced colon cancer. Aberrant crypts can be identified by their increased size, thicker epithelial lining, and increased pericryptal zone (2–8).

Few experiments have been designed to determine whether the early detection of ACF corresponds to the later development of tumors (7, 9–13), and very few studies have been performed to elucidate the effect of dietary fiber on aberrant crypt formation (13, 14).

To investigate whether the intake of different levels of the dietary fiber in our previous study design was reflected in the presence of ACF, archived colon tissue from all animals in the previous study (1) was examined.

Materials and Methods

Animals

Three-week-old male Wistar rats [Mol:WIST(SPF), Møllegård Breeding Center, L1. Skensved, Denmark] were assigned to nine groups after a two-week acclimatization period, according to Table 1.

Experimental Design

The animals were dosed with DMH-2HCl (20 mg/kg body wt; Sigma Chemical, St. Louis, MO) or solvent (0.9% NaCl solution) by gavage (5 ml/kg body wt) once a week for 10 weeks. Throughout the experiment (1 yr), the rats were given a powdered semisynthetic casein diet (15) in which the carbohydrates (cornstarch, potato starch, dextrin, and sucrose) were substituted with a dietary fiber (Fibeta) as the only source of fiber. The levels of Fibeta in the diets offered to the rats at the different periods during the study are presented in Table 1. The levels of Fibeta correspond to 0.7% and 14% dietary fiber in the rat diet. Fibeta consists of approximately 70% dietary fiber, 9% protein, 5% sucrose, 1% starch, 1% fat, and 5% ashes and water. A detailed description of the experimental condition and design, including analysis of the dietary fiber, has been given previously (1).

After one year, all animals were necropsied, and selected organs were fixed in 4% neutral buffered formaldehyde for histopathological examination. Before fixation, the large intestine was cut longitudinally, rinsed in 0.9% NaCl solution, cut into four pieces, and pinned on a cork slab. The divisions were made in the light of anatomic conditions based on the principles outlined by Lindström (16): the proximal colon (7 cm), the major flexure (5 cm), the distal colon (6 cm), and the residual colorectal segment (which varied in length).

Table 1. Experimental Design

Group No.	n^a	% Fibeta in Diet			DMH-2HCl, [b] mg/kg Body Wt
		8 Wks Preinitiation	10 Wks Initiation	30 Wks Postinitiation	
I	60	1	1	1	0
II	30	1	1	1	20
III	30	20	1	1	20
IV	30	20	20	1	20
V	30	20	20	20	20
VI	30	20	20	20	0
VII	30	1	20	1	20
VIII	30	1	1	20	20
IX	30	1	20	20	20

a: No. of rats.
b: Dimethylhydrazine dihydrochloride (DMH-2HCl) was given once a week for 10 wks.

Identification and Quantification of ACF

The histopathological data from the previous study revealed that the majority of the induced tumors were located in the proximal colon and major flexure (1). Inasmuch as other studies at our institute show that ACF are not seen in the proximal colon at different times after DMH initiation, the major flexure was selected for the examination for ACF. The middle 1.5 cm was selected for scoring. Giemsa stain (6 ml of concentrate in 50 ml of phosphate-buffered saline, pH 7.1, for 30 min) was used to visualize the ACF, and excess stain was rinsed off with phosphate-buffered saline. The tissue was placed luminal side up in a Petri dish with enough phosphate-buffered saline to cover the tissue, because we experienced that this procedure reduced the reflected light and made the scoring easier. The number of ACF and the number of aberrant crypts per focus were recorded blindly using a stereo microscope at ×40 magnification. The aberrant crypts were distinguished by their increased size, thicker and deeply stained epithelial lining, and increased pericryptal zone compared with normal crypts (4). An ACF may consist of one to several crypts, and in the present study the ACF were grouped into small (1–3 crypts), medium (4–6 crypts), and large (≥7 crypts) foci, respectively.

Statistics

Fisher's exact (2-tailed) test was used to compare the incidence of ACF between groups. Because of lack of normality of the data (Shapiro-Wilk), the comparison of total number and distribution of ACF with various numbers of crypts between groups was conducted by Wilcoxon rank-sum test when two levels were compared and Kruskal-Wallis test when more levels were compared. A probability of ≤5% was considered significant. All statistical calculations were carried out using SAS Release 6.03 (1988).

Results

Body weight, food consumption, clinical appearance, and tumor incidence data have been reported previously (1). Neither ACF nor tumors were observed in animals given 0.9% NaCl solution (Groups I and VI, data not shown). The values in Table 2 show a statistically significant lower number of rats with ACF in groups fed the high-fiber diet for 40 or 48 weeks than in groups fed the high-fiber diet for 0, 8, or 18 weeks.

The total number of ACF and the distribution of small, medium, and large foci per affected animal are presented in Table 3. Statistically significant differences in total number and number of small foci between some of the groups were seen. When pairwise comparisons of

Table 2. Influence of High-Fiber Diet on Number of Rats With ACF and Tumors and Comparison of Outcome of ACF and Colon Tumors[a–d]

Group No.	*n*	Total Time on HFD, Wks	No. of Animals With		
			ACF	Tumor	ACF and tumor
II	30	0	20 (67)	8 (27)	3
III	30	8	23 (77)	7 (23)	6
IV	29	18	23 (79)	6 (21)	6
V	30	48	11 (37)*	9 (30)	2
VII	30	10	15 (50)	9 (30)	8
VIII	29	30	12 (41)	8 (28)	3
IX	29	40	8 (28)*	4 (14)	0

a: All animals were dosed with DMH-2HCl.
b: Abbreviations are as follows: HFD, high-fiber diet; ACF, aberrant crypt foci.
c: Values in parentheses are percent.
d: Statistical significance is as follows: *, different from Groups II, III, and IV (Fisher's exact test, $p \leq 0.05$).

Table 3. Influence of High-Fiber Diet on Number of ACF in Rats and Distribution of ACF According to Number of Crypts in Focus

Group No.	n^a	No. of Foci/Colon[b–d]			
		Total	Small	Medium	Large
II	20	11.2 ± 2.6	6.0 ± 1.7	3.8 ± 0.9	1.4 ± 0.5
III	23	9.3 ± 2.5	5.8 ± 1.6	2.3 ± 0.8	1.3 ± 0.3
IV	23	10.5 ± 2.5	5.8 ± 1.4	2.9 ± 0.8	1.7 ± 0.5
V	11	4.4 ± 1.1*	1.7 ± 0.4*	1.4 ± 0.4	1.3 ± 0.4
VII	15	3.3 ± 1.9*	2.0 ± 1.3*	0.9 ± 0.5*	0.4 ± 0.2
VIII	12	6.3 ± 2.2	3.3 ± 1.1	2.0 ± 0.8	1.0 ± 0.5
IX	8	3.8 ± 1.4*	2.0 ± 0.9*	1.3 ± 0.5	0.5 ± 0.3

a: No. of affected animals.
b: Values are means ± SE.
c: Small foci, 1–3 crypts; medium foci, 4–6 crypts; large foci, ≥7 crypts.
d: Statistical significance is as follows: *, significantly less than Group II (Wilcoxon rank-sum test, $p \leq 0.05$).

number of total and small foci were made between groups, statistically significant differences were observed between rats fed low-fiber diet and those fed high-fiber diet for 10, 40, and 48 weeks, respectively.

It appears from Table 2 that no consistency exists between the presence of ACF and colon tumors. On the basis of individual animal findings, no correlation could be established between high numbers of ACF and presence of colon tumors (data not shown).

Discussion

In the previous study, no effect of the dietary fiber was found at any stage (preinitiation, initiation, and postinitiation) of the development of DMH-induced colorectal cancer (1). When the archived colon tissue from the same study was analyzed for ACF, a modulating effect of the dietary fiber was observed, and the data showed a correlation between intake of high-fiber diet and the presence of DMH-induced ACF.

In general, the values showed an inverse relation between duration of intake of high-fiber diet and incidence of rats with ACF as well as total number and number of small ACF per affected animal. The data for Group VII, those who received the high-fiber diet only during the 10-week initiation period, do not follow the pattern of the data for the other groups in relation to duration of intake of high-fiber diet. No plausible explanation can be given. If the data reflect a specific effect of dietary fiber when given during the initiation period, a similar effect would have been expected for Group IV, who received high-fiber diet in the preinitiation and initiation periods.

The ACF found in this experiment cannot directly be characterized as a precursor of colon cancer, inasmuch as the experimental design did not include interim sacrifice. However the DMH-induced ACF in the present study are similar to those found in other studies at our institute as well as those described in the literature as possible early markers of colon cancer.

Two other studies of ACF in relation to dietary fibers showed a reduction in ACF after feeding azoxymethane-induced rats flaxseed supplementation (14) or a low-risk diet that was high in fiber and calcium and low in fat (13). A third study with a different complex carbohydrate mixed in a Western-style diet showed that starch had no influence on the total number of foci induced by DMH but was able to reduce the number of affected crypts per focus (17).

Accumulating evidence supports the hypothesis that the development of ACF in laboratory animals and humans is indicative of a potential carcinogenic effect in the large bowel (18–21). The previously reported data from our experiment showed that a continuous feeding of rats

with a high-fiber diet resulted in increased production of intestinal volatile fatty acids, especially butyrate (1). Thus the demonstrated increase in production of butyrate and the reduction in ACF could indicate a protective effect of the beet fiber on the development of colon cancer in rats. However, these findings were not reflected in the colon tumor incidences previously obtained (1).

It has been proposed that ACF represent a preneoplastic lesion of colon cancer (2–8), and recent studies indicate an activation of the *ras* oncogene in azoxymethane-induced ACF and adenocarcinomas in rats, which points toward a preneoplastic nature of the ACF (22,23). The results from the present study, when assessed in terms of individual animal findings, did not show any correlation between occurrence of ACF and tumors. Only part of the major flexure of colon has been scored for ACF in this study. However, data from experiments at our institute have shown that the majority of the ACF are located in the major flexure. In addition, it should be borne in mind that the data derive from a large number of animals.

The distribution of ACF (Table 3) indicates that the differences in total number of ACF, to a great extent, are reflected in the number of small foci (1–3 crypts), which constitute the major part of the ACF. A minor change in medium-sized ACF (4–6 crypts) similar to that of the small foci is seen as well. Our studies did not reveal any intergroup difference in number of large foci or tumor incidence of colon. Earlier studies (7,12) showed that tumor development was correlated only with the larger foci and not with the total number of ACF. The lack of correlation between ACF and tumors in our study could be related to the observation that a statistically significant difference between groups was seen only in the number of total and small foci.

The hypothesis that ACF are preneoplastic lesions needs to be supported by further experimental data. The present state of knowledge could indicate that ACF represent either true preneoplastic lesions progressing into colon tumors or that ACF and colon tumors represent two parallel independent events as a consequence of the cancer initiation (i.e., the ACF not being preneoplastic lesions per se).

New studies are in progress in our laboratory to further elucidate the modulating effect of different diets by sequential analysis of selected intermediate markers in relation to development of colon cancer.

Acknowledgments and Notes

The authors thank Heidi Rokkedahl for excellent technical assistance. Address reprint requests to Dr. Inger Thorup, Institute of Toxicology, National Food Agency, 19, Mørkhøj Bygade, 2860 Søborg, Denmark.

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PAPER II

Influence of Different Diets on Development of DMH-Induced Aberrant Crypt Foci and Colon Tumor Incidence in Wistar Rats

Eva Kristiansen, Inger Thorup, and Otto Meyer

Abstract

The present study was undertaken to investigate certain dietary factors known to affect the development of colon cancer for their ability to modulate aberrant crypt foci (ACF). Male Wistar rats were initiated with oral doses of dimethylhydrazine dihydrochloride (DMH-2HCl, 20 mg/kg body wt) once a week for 10 or 20 weeks. Throughout the study the animals were fed 1) semisynthetic casein-based control diet, 2) control diet with 20% lard, 3) control diet with 20% lard and 20% dietary fiber, or 4) control diet where most of the carbohydrate pool was substituted with sucrose and dextrin. The composition of the different diets was designed to achieve equivalent intakes of essential nutrients. Animals were killed after 10, 20, and 31 weeks.

The study showed a pronounced effect of dietary composition on the development of DMH-induced ACF. The diet high in sucrose and dextrin caused a statistically significant increase ($p \leq 0.05$) in the total number of ACF and number of small and medium ACF. Adding lard to the standard diet did not cause an increase in ACF, but if the dietary fiber was added to the high-fat diet, a statistically significant reduction ($p \leq 0.05$) in the total number of ACF and number of small and medium ACF was observed. The values of large and extra-large foci reflected the same effect of diets on ACF.

The results indicate that tumors in the group fed the diet high in refined carbohydrates were more prominent and occurred with a higher incidence. However, the difference is based on few tumors and is not statistically significant. Our results do not show that the number of ACF and crypt multiplicity are conclusively predictive for tumor outcome with the present protocol, which did not include parameters to differentiate between ACF at the cellular level.

*(Nutr Cancer **23**, 151–159, 1995)*

Introduction

Accumulating evidence supports the hypothesis that the development of aberrant crypt foci (ACF) in laboratory animals and humans is indicative of a potential carcinogenic effect in the large bowel (1–4).

It has been proposed that ACF represent a preneoplastic lesion of colon cancer (5–11), and the ACF assay has been used by several investigators to study dietary modulators of colon carcinogenesis (8,12–18).

Previously, we reported an inverse relation between intake of a high-fiber diet and the incidence of dimethylhydrazine (DMH)-induced ACF (19). However, no effect of the dietary fiber was found at any stages of the development of colorectal cancer (20).

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The objective of the present study was to further investigate the effect of the dietary fiber on the ACF outcome and the correlation between ACF and tumor incidence in DMH-induced Wistar rats. In addition, the effects of other dietary macronutrients were investigated. Three different diets were tested: 1) a high-fat–low-fiber diet corresponding to the diet consumed by the Western populations with a high incidence of colorectal cancer, 2) a high-fat–high-fiber diet, and 3) a diet rich in sucrose and dextrin, reflecting the relatively high content of refined carbohydrates in the Western diet.

The experimental design, where the respective diets were fed during and after dosing of DMH, reflects the consumer situation where the initiation and promotion often will take place simultaneously.

Materials and Methods

Animals

One hundred thirty-two three-week-old male outbred rats [Mol:WIST(SPF)] were purchased from Møllegård Breeding Center (Ll. Skensved, Denmark).

Diets

The composition of the different diets is given in Table 1. Animals in Group I received the basic semisynthetic diet based on the formula given by Meyer and co-workers (21). In the Group II diet, the carbohydrate pool was reversed from 90% starches and 10% dextrin and sucrose to 10% and 90%, respectively. In Groups III and IV, animals received the basic diet in which 20% of the carbohydrate pool was replaced by lard (Group III) or 40% was replaced by 20% lard and 20% beet fiber (Group IV). The latter diets were adjusted to provide the animals in all groups with equal dietary intake of protein, vitamins, and minerals per calorie diet.

The beet fiber (Fibeta, De Danske Sukkerfabrikker, Nakskov, Denmark) was identical to that used in earlier studies (19,20), and the 20% corresponds to 14% dietary fiber in the rat diet. The fat was fully refined lard (Dafoma, Unilever, Sønderborg, Denmark).

Table 1. Composition of Diets

	Group			
	I	II	III	IV
Na-caseinate, %	20	20	26	25
Carbohydrate mix 1,[a] %	67		42	23
Carbohydrate mix 2,[b] %		67		
Soya bean oil (with vitamins A, D, and E), %	4	4	5	5
Mineral mixture, %	3.3	3.3	4.3	4.1
Vitamins B and K, choline chloride, inositol, and methionine, %	1.4	1.4	1.8	1.7
Cellulose, %	4	4	1.4	2
Lard, %			20	20
Fibeta,[c] %				20
Caloric density,[d] kcal/g	3.9	3.9	5.0	4.7

a: Carbohydrate mix 1: 45% cornstarch, 45% potato starch, 5% sucrose, 5% dextrin.
b: Carbohydrate mix 2: 5% cornstarch, 5% potato starch, 45% sucrose, 45% dextrin.
c: For specification see Ref. 20.
d: Calculation is as follows: fat = 9 kcal/g, protein = 4 kcal/g, carbohydrate = 4 kcal/g, Fibeta = 3 kcal/g.

Chemical

1,2-Dimethylhydrazine dihydrochloride (DMH-2HCl) was obtained from Sigma Chemical (St. Louis, MO).

Housing

The animals were kept in disposable plastic cages with an inserted steel grid floor, two animals per cage, in flexible film isolators (Isotec 12134, Olac, Oxford, UK) during the 1-week predosing period, the 10- or 20-week dosing period, and 2 weeks after termination of the dosing with DMH-2HCl. For the remaining period of the study, the animals were kept in stainless steel wire cages, two animals per cage. During the study, the temperature was maintained at 22 ± 1°C and relative humidity at 55 ± 5%, air was changed 8–10 times/hr, and fluorescent light was on from 2100 to 0900.

Experimental Design

The animals were randomly assigned to the four experimental groups. The animals were dosed with DMH-2HCl (20 mg/kg body wt) by gavage (5 ml/kg body wt) once a week for 10 weeks (10 animals/group) or 20 weeks (18 or 28 animals/group) according to Table 2.

Body weight and food and water consumption were measured weekly. Interim sacrifices were performed after 10 weeks (10 animals/group) and 20 weeks (8 animals/group). The rest of the animals were sacrificed after 31 weeks. Complete gross necropsy was performed on all animals, and the large intestine was examined histopathologically. Before fixation, the large intestine was cut longitudinally, rinsed in 0.9% NaCl solution, and divided into four pieces. The first segment comprised the proximal colon with the herring bone mucosal structure; the remaining part was divided in three parts of equal size. The colon segments were pinned on a cork slab and fixed in 4% neutral buffered formaldehyde. After fixation, Giemsa stain (6 ml of concentrate in 50 ml of phosphate-buffered saline, pH 7.1, for 15 mins) was used to visualize the ACF, and excess stain was rinsed off with phosphate-buffered saline. The tissue was placed luminal side up in a petri dish with enough phosphate-buffered saline to cover the tissue. The number of ACF and the number of aberrant crypts per focus were recorded using a stereomicroscope at ×40 magnification. The aberrant crypts were distinguished by their increased size, thicker and deeply stained epithelial lining, and increased pericryptal zone compared with normal crypts (7). An ACF may consist of one to several crypts, and in the present study the ACF were grouped into small (1–3 crypts), medium (4–6 crypts), large (7–9 crypts), or extra-large (≥10 crypts) foci.

Colon tissue deviating from normal morphology at gross examination was embedded in paraffin, sectioned (4–6 μm), and stained with hematoxylin and eosin for microscopic examination. The size and distribution of all colon tissue changes were registered.

Table 2. Experimental Design[a,b]

Group	10 Wks Initiation	20 Wks Initiation	20 Wks Initiation + 11 Wks Postinitiation
I	10	8	10
II	10	8	10
III	10	8	20
IV	10	8	20

a: Dimethylhydrazine dihydrochloride (DMH-2HCl), 20 mg/kg body wt/wk.
b: Values are expressed as no. of rats.

Statistics

One-way analysis of variance was used to analyze body weight, weight gain, and food and water consumption. A test for normal distribution (Shapiro-Wilk) of ACF data was carried out on logarithmically transformed data. Two-way analysis of variance (diet × time) on the logarithmically transformed data was used to determine the effect of diet on the ACF over all time points. (One-way analysis of variance was used for analyzing each time point separately.) Duncan's multiple range test was used for pairwise comparisons. Fisher's exact (2-tailed) test was used to determine the effect of diet on tumor incidence. A probability of ≤5% was considered significant. All statistical calculations were carried out using SAS release 6.04.

Results

Body Weight and Food and Water Consumption

During the experiment, no treatment-related signs of adverse effect in clinical appearance of the animals were observed. A statistically significant increase in average body weight during the entire experimental period was seen in Group III animals offered the 20% fat diet compared with the other groups. No other treatment-related changes were observed in body weights. The data for food consumption (not shown) revealed a consistently lower average weekly intake during the entire experimental period for animals fed the 20% lard diet (Groups III and IV) and a lower average weekly intake during the first one-third of the study for animals fed the diet low in starches and high in dextrin and sucrose (Group II) than for Group I. The lower values for average weekly food intake for Group II were reflected in the values for average energy intake in kilocalories per animal per week (3–16% lower than Group I). The corresponding values for Group IV animals were statistically significantly lower in a few experimental weeks than for Group III (<10% apart from Weeks 23 and 24, where values were 15% and 11% lower, respectively). A comparison of the data for Groups I and III revealed no consistent difference in average energy intake. No consistent treatment-related changes were observed in water consumption.

ACF

The total number of ACF and distribution of ACF are presented in Table 3. Two-way analysis of variance demonstrated a significant effect of diet on total number of ACF and number of small and medium ACF over all time points. Significant effect of time was seen with respect to total number of ACF and number of medium ACF. Because of a small number of large and extra-large foci in some of the groups, lack of normality of these data was found, and therefore analysis of variance was not applied, but the effect of diet and time on these ACF was the same (Table 3). The total number of ACF and distribution of ACF between groups showed the same effect with respect to diet when analyzed at each time point (data not shown).

Highly refined carbohydrates (Group II) significantly increased the number of ACF, whereas the dietary fiber (Group IV) caused a significant decrease in ACF. A schematic illustration of development of ACF with respect to diet and time is given in Figure 1. From Table 3, it appears that the relative distribution of small, medium, large, and extra-large ACF is similar among groups.

Tumors

No tumors were observed 10 weeks after the start of dosing (data not shown). Tumors were observed 20 and 31 weeks after the start of dosing, but no statistically significant difference in incidence between groups was seen (Table 4). No tumors were observed in animals given

Table 3. Influence of Different Diets on Number of ACF in Rats and Distribution of ACF According to Number of Crypts in Focus

Wks	*n*	Group	ACF/Colon[a–c] Small	Medium	Large	X-large	Total
10	10	I	70.5 ± 18.8 (87)	9.5 ± 4.2 (12)	0.8 ± 0.2 (1)	0.0 ± 0.0 (0)	80.8 ± 22.8
	10	II	197.0 ± 36.3 (91)	18.6 ± 4.9 (8)	1.1 ± 0.4 (1)	0.2 ± 0.1 (0)	216.7 ± 39.6
	10	III	109.6 ± 49.1 (93)	7.2 ± 4.7 (6)	0.6 ± 0.5 (1)	0.0 ± 0.0 (0)	117.4 ± 53.6
	10	IV	40.7 ± 12.9 (90)	4.2 ± 1.5 (9)	0.3 ± 0.2 (1)	0.1 ± 0.1 (0)	45.2 ± 14.2
20	8	I	108.0 ± 21.7 (81)	21.1 ± 5.5 (15)	3.5 ± 1.3 (3)	0.9 ± 0.5 (1)	133.5 ± 26.8
	8	II	366.1 ± 72.6 (69)	121.3 ± 29.4 (23)	27.0 ± 10.2 (5)	13.6 ± 9.5 (3)	528.0 ± 113.4
	8	III	102.0 ± 37.9 (88)	13.0 ± 4.5 (11)	0.9 ± 0.4 (1)	0.4 ± 0.2 (0)	116.3 ± 42.7
	8	IV	48.9 ± 30.0 (82)	10.3 ± 6.9 (17)	0.6 ± 0.4 (1)	0.3 ± 0.2 (0)	60.0 ± 37.4
31	9	I	110.7 ± 31.2* (66)	42.8 ± 9.1* (26)	10.4 ± 1.9 (6)	3.0 ± 0.8 (2)	166.9 ± 40.0*
	9	II	318.2 ± 64.9† (66)	120.9 ± 25.8† (25)	28.9 ± 7.2 (6)	11.8 ± 4.6 (3)	479.8 ± 89.2†
	18	III	122.7 ± 35.2* (79)	26.6 ± 5.1* (17)	5.3 ± 1.1 (3)	1.5 ± 0.6 (1)	156.1 ± 40.4*
	20	IV	50.6 ± 15.4‡ (67)	19.3 ± 8.0‡ (25)	4.7 ± 2.0 (6)	1.6 ± 0.6 (2)	76.1 ± 25.6‡
			Statistical significance (2-way analysis of variance, P values)				
Diet			0.0001	0.0001			0.0001
Weeks			0.2057	0.0001			0.0134
Weeks × diet			0.8460	0.2702			0.7773

a: Values are means ± SE; nos. in parentheses represent percentage of total; *n,* effective no. of rats. ACF, aberrant crypt foci.
b: Small foci, 1–3 crypts; medium foci, 4–6 crypts; large foci, 7–9 crypts; X-large foci, ≥10 crypts.
c: Groups not sharing a common superscript (*, †, ‡) differ significantly by Duncan's multiple range test ($p \leq 0.05$).

Table 4. Influence of Different Diets on Incidence of Colorectal Tumors in Rats[a]

Group	Time After Dosing 20 Wks	31 Wks	Total ~~No. of Tumors~~
I	2/8 (25)	4/10 (40)	6/18 (33)
II	3/8 (38)	8/10 (80)	11/18 (61)
III	1/8 (13)	12/20 (60)	13/28 (46)
IV	0/8 (0)	14/20 (70)	14/28 (50)

a: Values are expressed as no. of affected animals/total no. of animals; values in parentheses are percentages.

the high-fiber diet at 20 weeks. At both sacrifices, the highest tumor incidence was seen in Group II, in which a greater part of the tumors appeared large (≥10 mm diam), reddish, and cauliflower-like. When assessed on the basis of individual animal findings, no correlation between occurrence of ACF and tumors (data not shown) could be found.

Discussion

The composition of the diets was designed to achieve equivalent intakes of essential nutrients on the basis of the assumption that rats would consume equivalent energy from each of the diets, even though concentrations of macronutrients would differ. Irrespective of equivalent energy intake for Groups I and III, the weight gain of the animals in the latter group fed 20% lard was significantly higher. This observation is in accordance with that of Malville-Shipan and Fleming (22). The lower energy intake in the animals fed the diets high in dextrin and sucrose (Group II) or the 20% beet fiber diet (Group IV) did not result in lower weight gain than in those receiving the basic diet (Group I) and was not considered to interfere with the nutrient requirement of the animals.

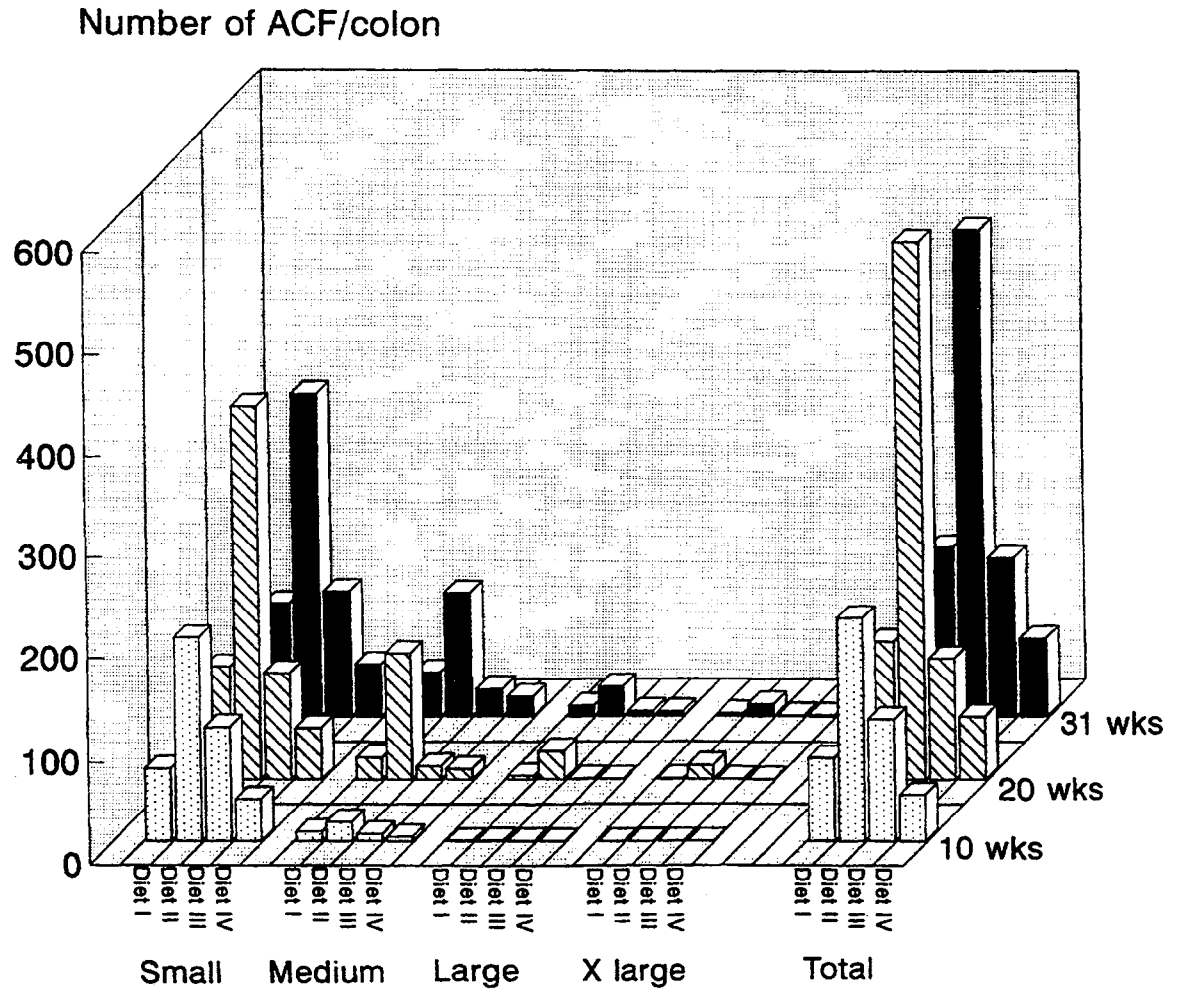

Figure 1. Influence of different diets on development of dimethylhydrazine-induced aberrant crypt foci (ACF) in rats at 3 time points. Diet I, basic diet; Diet II, high-sucrose/dextrin diet; Diet III, high-fat diet; Diet IV, high-fat/high-fiber diet. Small foci, 1–3 crypts; medium foci, 4–6 crypts; large foci, 7–9 crypts; X-large foci, ≥10 crypts.

The present investigation showed that a diet high in refined carbohydrates caused a statistically significant increase in the number of ACF. Adding lard to the standard diet did not cause an increase in ACF, but if the beet fiber was added to the high-fat diet, a statistically significant reduction in ACF was observed.

High content of refined carbohydrates caused a statistically significant increase in total number of ACF and number of small and medium ACF. The high-fat diet did not affect the number of ACF, inasmuch as no statistically significant difference could be detected between Groups I and III. Adding dietary fiber (Group IV) to the high-fat diet (Group III) caused a statistically significant decrease in total number of ACF and number of small and medium ACF. Even though statistical analysis was not applied to the data on large and extra-large foci, the values reflect the same effect of diets on ACF.

In Group I, the carbohydrate pool mainly consisted of starches (Table 1). Analysis showed that approximately 55% of the potato starch and 7% of the cornstarch was resistant starch (23), which physiologically may act as a dietary fiber. The smaller number of ACF in Group I than in Group II could be interpreted as a consequence of a fiber-like effect of starches similar to the effect obtained by adding dietary fiber to the diet in Group IV. It could be speculated that the effect is caused by the specific fiber used or that the effect is a consequence of the lower dietary intake of calories of the high-fiber fed animals. However, the latter

explanation is not supported by the data for Group II animals having a high number of ACF irrespective of the relatively low caloric intake.

The observed modulation of refined carbohydrates on development of ACF is in accordance with results obtained by other investigators. In DMH-induced rats, Caderni and associates (12) found that a high-sucrose diet caused a significant increase in the percentage of large ACF (3–6 crypts) and a reduction in percentage of small ACF (1–2 crypts) compared with a high-starch diet. Stamp and colleagues (24) compared the effect of certain refined sugars (sucrose, glucose, fructose) on ACF in azoxymethane (AOM)-induced mice. The results demonstrated that oral gavage of sucrose and fructose led to a significant increase in number of ACF, whereas glucose did not. Furthermore case-control studies indicate that refined sugar may be a risk factor for human colorectal cancer (25–29).

Previously, we showed a statistically significant inverse relation between duration of intake of a high-fiber diet and number of animals with ACF as well as the total number of ACF and number of small ACF (1–3 crypts) per affected animal (19). Similar results have been obtained by others. A reduction in ACF after feeding AOM-induced rats flaxseed supplementation (16) or a low-risk diet high in fiber and calcium and low in fat (15) has been shown. In a study by Hardman and others (14), DMH-induced rats fed diets with pectin and corn oil added had significantly fewer total ACF and medium ACF (4–6 crypts) per rat than rats fed a fiber-free diet with no corn oil added.

The present study did not support the modulating effect of a high-fat diet on the development of ACF shown in other studies. Shivapurkar and co-workers (15) showed that AOM-induced rats on a high-risk diet, high in fat and low in fiber and calcium, had a higher number of ACF than those fed a low-risk diet. Similar findings have been reported by McLellan and Bird (8), who found that AOM-induced mice fed a high-fat diet had more ACF per colon and larger mean size of the ACF than those fed a low-fat diet.

The relative distribution of ACF categorized by crypt multiplicity is similar between groups, whereas the absolute number of ACF for all categories is very much higher in Group II. The DMH dose regimen employed in the present study implies that the data mirror the influence of the different diets on the development of ACF and tumor during initiation and promotion. This regimen reflects the consumer situation, in which initiation and promotion take place simultaneously.

The number of small ACF increases after 10 and 20 weeks in all groups, revealing a constant induction of small ACF during the 20 weeks of DMH administration. The corresponding values for the medium, large, extra-large, and total ACF show a constant increase in number during the entire period except in Group II. The data concerning Groups I, III, and IV indicate a continuous development in size of ACF. The different development pattern of medium, large, and extra-large ACF in Group II animals receiving the diets high in sucrose and dextrin could be hypothesized as being a result of a cocarcinogenic effect of sucrose and dextrin on the DMH induction of ACF and consequently an initial induction of a very high number of ACF, leading to a biologic saturation of the capacity of the intestinal epithelium for further development of ACF.

No statistically significant differences in tumor outcome (benign or malignant) were seen among the groups. The most prominent tumors were seen in Group II, in which the number of aberrant foci with four or more crypts was very high. Earlier studies (10,30,31) found crypt multiplicity to be a better predictor of tumor outcome than the total number of ACF. Even though our results indicate a correlation between ACF and tumors, they are not able unequivocally to support the theory that the presence of a high number of ACF with high crypt multiplicity is predictive for tumor outcome. The absence of correlation might be related to the rather low tumor incidence.

When assessed on individual animal findings, no correlation between occurrence of ACF and tumors could be found. In fact, some of the most prominent tumors were found among

animals with no or few aberrant foci with four or more crypts. That the number of ACF and crypt multiplicity were not predictive for tumor outcome has also been reported by Hardman and co-workers (14).

In conclusion, the study showed that the dietary composition has a pronounced effect on development of DMH-induced ACF. Even though the tumors in Group II were more prominent and occurred with somewhat higher incidence, the overall tumor picture did not unequivocally support the ACF data. In addition, there was a lack of correlation between the occurrence of ACF and tumors in the individual animals.

Recent investigations (32–34) revealed ACF as a heterogeneous group of lesions. The present study did not include parameters to differentiate between ACF at the cellular level, but our results do not show that the number of ACF and crypt multiplicity are conclusively predictive for tumor outcome.

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PAPER III

ANTICANCER RESEARCH *15*: 2101-2106 (1995)

Effect of Potato Starch, Cornstarch and Sucrose on Aberrant Crypt Foci in Rats Exposed to Azoxymethane

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Abstract. *Studies have shown that different kinds of carbohydrates are able to modify the development of colo-rectal cancer in animals as well as humans. In the present study with rats sucrose and two types of starches were investigated for their effects on the development of aberrant crypt foci (ACF), which have been proposed to represent preneoplastic lesions of colo-rectal cancer. Fifty-six three-week-old male Wistar rats were randomly assigned to four groups and dosed subcutaneously with AOM (15 mg/kg body wt) once a week for 2 weeks. At the end of the dosing period the animals were allocated to their respective diets. Group I was fed the basic diet; in Group II the carbohydrate pool in the diet was replaced by sucrose, in Group III by potato starch and in Group IV by cornstarch. Animals receiving the potato starch diet showed a statistically significant reduction in body weight gain. A statistically significantly lower number of ACF in all categories but small were demonstrated in animals given potato starch, and in addition an effect was seen in the relative distribution of ACF with fewer of the larger ACF. No effect of sucrose or cornstarch was seen. Explanations of the inhibitory effect in the potato starch group on the development of ACF could either be the lower daily caloric intake or the substantial amounts of resistant starch in the potato starch used.*

Studies during the last years have shown that a significant amount of starches escapes digestion in the small intestine (1-3). These starches, called resistant starches, may reach the large intestine and are here available for fermentation like dietary fibres. Like dietary fibres, resistant starches may be important as a protective factor in human colo-rectal cancer (4-6). In contrast to this effect, recent data have shown that refined sugar may be a risk factor for development of this lesion (7-11).

Experimental studies have shown that cornstarch may inhibit some of the steps in intestinal carcinogenesis in azoxymethane-induced rats (12) and decreases the dimensions of dimethylhydrazine-induced aberrant crypt foci (ACF) in the colon (13). ACF have been proposed to represent preneoplastic lesions of colo-rectal cancer (14-20). Previous studies from our laboratory have shown that a diet rich in refined carbohydrates increases the number and/or size of aberrant crypt foci (ACF), whereas a high-fibre diet showed the opposite effect (21,22). The inhibitory effect of dietary fibre on the formation of ACF has also been demonstrated by Alabaster *et al* (23).

In the present study we have investigated the effect of sucrose and two different types of starches on the development of azoxymethane-induced ACF in male Wistar rats. The starches tested were a potato starch with very high amounts of resistant starch and cornstarch with only low amounts.

Materials and Methods

Animals. Fifty-six three-week-old male outbred rats [Mol:WIST(SPF)] were obtained from Møllegård Breeding Center, (Ll. Skensved, Denmark).

Diets. The composition of the different diets is given in Table I. Animals in Group I received the basic semisynthetic diet based on the formula given by Meyer *et al* (24). In the Group II diet the carbohydrate pool was

Abbreviations: AOM, azoxymethane; ACF, aberrant crypt foci.

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replaced by sucrose, in Group III by potato starch and in Group IV by cornstarch.

Chemical. Azoxymethane (AOM) was obtained from Sigma Chemical (St. Louis, MO).

Housing. The animals were kept in disposable plastic cages with an inserted steel grid floor, two animals per cage, in flexible film isolators (Isotec 12134, Olac, Oxford, UK) during the 1-week predosing period, the 2-week dosing period, and for 2 weeks after termination of the dosing with AOM. For the remaining period of the study, the animals were kept in stainless steel wire cages, two animals per cage. During the study the temperature was maintained at 22 ± 1°C and relative humidity at 55 ± 5%, air was changed 8-10 times/hr, and fluorescent light was on from 2100 to 0900.

Experimental design. The animals were randomly assigned to the four experimental groups (14 animals per group) and after the predosing period AOM (15 mg/kg body wt) was administered subcutaneously once a week for 2 weeks. All animals were fed the group I basic diet during the predosing and dosing period and then allocated to their respective diets.

Body weight and food consumption were measured weekly. The animals were sacrificed 18 weeks after the first AOM-injection. Complete gross necropsy was performed on all the animals. The large intestine was cut longitudinally, rinsed in 0.9% NaCl solution, and divided into four pieces as described earlier (22). The colon segments were pinned on a cork slab and fixed in 4% neutral buffered formaldehyde. To visualize the ACF the colon segments were processed according to (22). The ACF were grouped into small (1-3 crypts), medium (4-6 crypts), large (7-9 crypts) or extra large (≥10 crypts) foci.

Colon tissue deviating from normal morphology at gross examination was embedded in paraffin, sectioned (4-6 μm) and stained with haematoxylin and eosin for microscopic examination.

Statistics. One-way analysis of variance was used to analyse body weight, weight gain and food consumption. A test for normal distribution (Shapiro-Wilk) of ACF data was carried out on logarithmically transformed data. One-way analysis of variance on the logarithmically transformed data was used to determine the effect of diet on the ACF. Duncan's multiple range test was used for pairwise comparisons. A probability of ≤5% was considered significant. All statistical calculations were carried out using proc GLM, SAS release 6.04.

Results

During the experimental period four animals from group III died accidentally from volvulus due to a torsion of the caecum about the ostia of the terminal ileum and proximal colon. No other treatment-related signs of clinical adverse effects on the animals in the different groups were observed during the experiment. However, the animals receiving the diet with potato starch as the only carbohydrate source were smaller, and showed a statistically significant reduction in body weight gain, compared with those in any other group after 5 weeks on the experimental diet (up to 28%). A concomitant decrease in food consumption was recorded in the potato starch fed animals, statistically significant compared to that of the other groups, from week 6 until week 11. The corresponding data for the relative food consumption in these animals showed a statistically significant increase from week 10 and onwards.

At necropsy a caecal enlargement was observed in group III. In addition, the livers from these animals appeared slightly pale and the lesions turned out to be diffuse fatty liver confined to the centrolobular zone. No signs of necrosis or fibrosis were seen.

Table I. *Composition of diets.*

Group	I	II	III	IV
Na-caseinate, %	20	20	20	20
Carbohydrate mix [a] %	67	0	0	0
Sucrose, %	0	67	0	0
Potato starch, %	0	0	67	0
Cornstarch, %	0	0	0	67
Soya bean oil (with vitamins A, D, and E), %	4	4	4	4
Mineral mixture, %	3.3	3.3	3.3	3.3
Vitamins B and K, choline chloride, inositol, and methionine, %	1.4	1.4	1.4	1.4
Cellulose, %	4	4	4	4

[a]: Carbohydrate mix: 45% cornstarch, 45% potato starch, 5% sucrose, 5% dextrin.

ACF. The total number and distribution of ACF are presented in Table II. In animals given potato starch a statistically significantly lower number of ACF in all categories but small was seen. With respect to small ACF, a statistically significant difference was seen between animals given potato starch and cornstarch.

In addition, an effect of potato starch was reflected in the relatively fewer large and extra large ACF compared to the other groups (Table II).

Compared to the standard diet sucrose and cornstarch did not show any effect on the development of ACF.

Tumors. Macroscopically visible tumors were seen in groups I and II. No tumors were seen among animals given potato starch or cornstarch (Table III).

Because of the low tumor incidence no statistical analysis was carried out. When assessed on individual animal findings, no correlation between number or crypt multiplicity of ACF and occurrence of tumors could be found (data not shown).

Discussion

The reduction in body weight gain in the group III animals receiving 67% potato starch compared to that of any of the other groups, including the group I receiving a carbohydrate mixture containing 30% potato starch, is considered to be consequence of the lower digestibility of the potato starch. Both the decrease in body weight gain and the concomitant increase in relative food consumption support previously reported data on potato starch (25-28).

Table II. *Effect of different diets on the number and distribution of ACF according to the various number of crypts in colons of Wistar rats given AOM.*

			No. of foci per colon [a,b,c]				
Wks	N[d]	Group	Small	Medium	Large	X large[e]	Total
18	14	I	$89.6\pm7.4^{X,Y}$ (52)	55.4 ± 3.9^{X} (32)	11.3 ± 1.7^{X} (7)	14.7 ± 2.3^{X} (9)	170.9 ± 11.6^{X}
18	14	II	$122.0\pm17.0^{X,Y}$ (68)	41.4 ± 6.1^{X} (23)	7.4 ± 1.2^{X} (4)	9.1 ± 1.6^{X} (5)	179.9 ± 24.2^{X}
18	10	III	85.0 ± 18.2^{Y} (73)	26.6 ± 6.6^{Y} (23)	2.1 ± 0.8^{Y} (2)	2.9 ± 1.1^{Y} (2)	116.6 ± 24.8^{Y}
18	14	IV	115.6 ± 10.3^{X} (61)	49.4 ± 6.5^{X} (26)	10.7 ± 2.7^{X} (6)	13.4 ± 3.5^{X} (7)	189.1 ± 18.0^{X}
Statistical significance (One-way analysis of variance, P- values)							
			0.1334	0.0036	0.0001	0.0001	0.0283

a: All the values shown are mean ± S.E., values in parentheses are percentages of total.
b: Small foci: 1-3 crypts; Medium foci: 4-6 crypts; Large foci: 7-9 crypts; X large foci: 10 or more crypts.
c: Groups not sharing a common superscript differ significantly by Duncan's multiple range test ($P \leq 0.05$).
d: N: effective number of rats.
e: X large: extra large.

The depression in body weight gain in the potato starch fed animals corresponds to that of rats on a moderately restricted (50%) diet reported by Levin (29).

Moderate dietary restriction *versus ad libitum* feeding in a toxicological study caused in general qualitatively the same toxic response in different parameters, although some quantitative differences were seen (30). Therefore it is considered that the imposed caloric restriction dit not impair the validity of the model used.

All animals in group III showed a caecal enlargement which is regarded as a consequence of the microbial fermentation of the high resistant potato starch leading to production of a lot of volatile fatty acids in the large intestine. The caecal enlargement together with the anatomic disposition (the close proximity of the ileum and the colon exit in caecum) and the free mobility of the caecum is probably the cause of the four deaths observed in group III.

The volatile fatty acids are readily absorbed and acetate and propionate are carried to the liver. Their actions here remain unclear (31), but an investigation by Yamamoto and Yamakava (32) has shown that 12 weeks' administration of acetate to rats caused fatty degeneration in the liver.

In rats fed potato starch a statistically significantly lower number of ACF in all categories but small were seen. Explanations of this could either be the lower daily caloric intake in the potato starch group or the substantial amounts of resistant starch in the potato starch used.

Lasko and Bird (33) have shown that caloric restriction affects the number of AOM-induced ACF in rats. Compared to ad libitum fed rats, late intervention with caloric restriction for 12 weeks reduced the total number of ACF. Also, the number of colonic adenomas was reduced by caloric restriction. With respect to tumor development,the inhibiting effect of caloric restriction has been known for a long time. In a recent commentary Rogers and co-workers (34) summarize the mechanisms postulated for the reduction of tumorigenesis by caloric restriction. Among these are: general reduction in growth of all tissues, due to reduced growth-promoting blood hormones; alteration of the metabolism of carcinogens; reduction in oxidative damage to DNA; and reduction of cell division, DNA synthesis, DNA adduct formation and alteration of DNA repair.

Table III. *Incidence of colorectal tumors in rats fed four different carbohydrate sources.*[a]

Group	No. of rats	Adenomas	Adenocarcinoma *in situ*	Total no. of tumors
I	14	3 (22)	1 (7)	4 (29)
II	14	2 (14)	0	2 (14)
III	10	0	0	0
IV	14	0	0	0

[a]: Values are expressed as no. of affected animals/total no. of animals, values in parentheses are percentages.

According to the method of Englyst *et al* (35), resistant starch constituted 55% of the potato starch used. The resistant starch may reach the large intestine and is here available for fermentation like dietary fibres. A few studies have shown that a high fibre diet reduced the number of ACF (21-23). Further, a case-control study by Marquart-Moulin *et al* (9) has revealed that a high intake of potatoes decreased the risk of cancer of the colon and rectum.

Moreover, it has been shown that resistant starch forms more butyrate than most types of dietary fibre (4,5,31,36) and that butyrate possesses anticarcinogenic properties (4,5,36,37). Studies have shown that human faecal suspensions were able to form high proportions of butyrate from potato starch (36,38). On the other hand, Mallett *et al*

(28) showed that, even though the total amount of volatile fatty acid in caecum was very high in rats fed potato starch, the main production was acetate.

In the present study the relative number of ACF with high crypt multiplicity was lower in the animals fed potato starch. Some studies (19,39-41) have found the number of ACF with high crypt multiplicity to be a better predictor of tumor outcome than the total number of ACF.

The cornstarch diet had no inhibitory effect on the formation of ACF. The reason might be that the cornstarch used only consists of 7% resistant starch according to the method of Englyst *et al* (35). Therefore only a minor amount of resistant starch reaches the large intestine for fermentation, *e.g.* it does not possess a substantial dietary fibre effect.

It has been shown that refined sugar may be a risk factor for the development of colo-rectal cancer in humans (7-11) and enhances the development of ACF (22). In the present study sucrose did not cause any increase in the formation of ACF.

Due to the short duration of the study only a few colon tumors were seen, but it is noticeable that no tumors were observed in animals given the high-starch diets. This observation supports the suggestion by Cassidy *et al* (6) and Marquart-Moulin *et al* (9) that starch plays an important role in protection against colo-rectal cancer in humans.

This study revealed a significant inhibitory effect of potato starch on the development of ACF. Whether this effect of the potato starch is an effect of the starch *per se* or an effect of the caloric restriction created has to be further investigated.

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PAPER IV

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The ability of two cooked food mutagens to induce aberrant crypt foci in mice

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The aberrant crypt foci assay has been used extensively to study different compounds for chemopreventive action, but almost all investigations have used initiators not normally found in the diet. In the present study two food-borne initiators, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) were used. To simulate the human exposure further, we chose a feeding regimen with continuous low IQ- and PhIP-doses. Throughout the study female mice were given diets with or without 0.03% IQ or 0.03% PhIP. Two additional groups were given azoxymethane (AOM) (5 mg/kg body weight) and 1,2-dimethylhydrazine dihydrochloride (DMH-2HCl) (20 mg/kg body weight), respectively, one dose a week for two weeks. Animals were killed after four and 10 weeks. After four weeks only the mice dosed with IQ and PhIP had aberrant crypt foci. A much higher number of aberrant crypt foci were found in the IQ mice (31.8 ± 5.2) than in the PhIP mice (0.5 ± 0.3). After 10 weeks aberrant crypt foci were found in all dosed groups. The IQ mice had significantly more ($P \leq 0.001$) small and total aberrant crypt foci than the other groups. AOM and DMH induced a higher percentage of medium or large sized aberrant crypt foci than PhIP or IQ. The interpretation of the aberrant crypt foci as precursor lesions for colon cancer in the PhIP and IQ mice is difficult because PhIP and IQ have not been reported to be colonic carcinogens. If cooked food mutagens such as IQ or PhIP are to be used as initiators in the aberrant crypt foci test, the use of rats may be preferable.

Key words: Aberrant crypt foci, initiators, IQ, mice, PhIP.

Introduction

With increasing demands for the testing of potential anticarcinogenic compounds in foods there is a need for models which produce test results within a few months. The aberrant crypt foci assay is a short-term model and evidence supports the hypothesis that the development of aberrant crypt foci in laboratory animals and people is indicative of a potential carcinogenic effect in the large bowel (Bird *et al*, 1989; Pretlow *et al*, 1991; Roncucci *et al*, 1991a,b). The assay has been used by several investigators to study chemicals and dietary components for chemopreventive action (reviewed by Bird, 1995a), but the published data almost exclusively deal with study designs where the initiators are not normally found in the diet. The two colon carcinogens 1,2-dimethylhydrazine dihydrochloride (DMH) or azoxymethane (AOM) have mostly been used. If food-borne initiators in the human diet could be used in the aberrant crypt foci assay, this test could be a very useful model to screen for the effects of proposed dietary anticarcinogens on colon carcinogenesis. Thus, we decided to test the aberrant crypt foci assay using two heterocyclic amines formed during heating of protein-containing foods, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP). To simulate further likely human exposure, we chose a feeding regimen with continuous low doses of IQ and PhIP.

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Both IQ and PhIP are mutagenic and carcinogenic in laboratory animals (Eisenbrand and Tang, 1993). Compounds like IQ and PhIP, and also probably many of the potentially anticarcinogenic compounds, are in short supply and often very expensive. Hence, the use of mice would obviously be more beneficial than rats. The only reported study on IQ- and PhIP-induced aberrant crypt foci in mice is a three-week study by Tudek *et al* (1989) in which a few aberrant crypt foci were observed after administration of two doses of IQ (total 400 mg/kg body weight) and PhIP (total 150 mg/kg body weight) over a period of three weeks. The aim of the present study was to evaluate the use of low doses of IQ or PhIP administered over 4–10 weeks to mice in the aberrant crypt foci assay and compare these with DMH and AOM.

Methods

Animals

Seventy four-week-old female mice C57BL/6J, specified as pathogen-free, were purchased from Bomholtgård Breeding and Research Centre Ltd, Denmark. The mice were randomly allocated by weight into the different groups (Table 1) and were individually marked by ear tags and tail marks.

Diets

The animals were offered a pellet diet of Altromin 1314. Altromin 1314 pellets with 0.03% IQ (group II) or 0.03% PhIP (group III), respectively, were prepared by Altromin GmbH u. Co KG, Lage, Lippe, Germany (Table 1). Analyses of IQ and PhIP in the two batches of prepared pellets were in accordance with the amount added.

Table 1. Allocation of mice into treatment groups

Group	Diet	Initiation at 2 weeks	No of Animals*
I	Altromin 1314	0	14
II	Altromin 1314 with 0.03% PhIP	0	14
III	Altromin 1314 with 0.03% IQ	0	14
IV	Altromin 1314	AOM 5 mg/kg bw/week	14
V	Altromin 1314	DMH 20 mg/kg bw/week	14

* 4 animals in 4 weeks, and 10 animals in 10 weeks.

The animals in groups IV and V were offered Altromin 1314 and dosed intraperitoneally (10 ml/kg body weight) with AOM (5 mg/kg body weight) and DMH-2HCl (20 mg/kg body weight), respectively, once a week for two weeks.

Chemicals

1,2-Dimethylhydrazine dihydrochloride (DMH-2HCl) and azoxymethane (AOM) were obtained from Sigma Chemical Co (St Louis, MO, USA). 2-Amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) were a generous gift from Dr Snorri S Thorgeirsson, National Cancer Institute, Bethesda, MD, USA.

Housing

The animals were kept in polycarbonate cages on beech bedding (Spanvall Red Special, Spanvall Jerslev, Denmark) with two females in each cage. The groups dosed with AOM and DMH-2HCl were kept in a flexible film isolator (Isotec 12134, Olac Inc, Oxford, UK) during the week before dosing started, during the 2 weeks of the dosing period, and for 2 weeks after termination of the dosing. During the study, the temperature was maintained at 22 ± 1°C and relative humidity at 55 ± 5%, air was changed 8–10 times/h, and fluorescent light was kept on from 2100 to 0900 h.

Experimental design

Body weight and food consumption were measured weekly. Interim sacrifices were performed 4 weeks after the first dose (4 animals/group) and the remaining animals were killed after 10 weeks (10 animals/group). Complete gross necropsy was performed on all animals, and the large intestine was examined microscopically. Before fixation, the large intestine was cut longitudinally and rinsed in 0.9% NaCl solution. The colon was then pinned on a cork slab and fixed in 4% neutral buffered formaldehyde. After fixation, Giemsa stain (6 ml of concentrate in 50 ml of phosphate-buffered saline (PBS), pH 7.1, for 15 min) was used to visualize the aberrant crypt foci and excess stain was rinsed off with PBS. The tissue was placed luminal side up in a Petri dish with enough PBS to cover the tissue. The total number of aberrant crypt foci and the number of aberrant crypts per focus were recorded using a stereomicroscope at × 40 magnification.

The aberrant crypts were distinguished by their increased size, thicker and deeply stained epithelial lining, and increased pericryptal zone compared with

normal crypts (McLellan and Bird, 1991). An aberrant crypt focus may consist of one to several crypts, and the aberrant crypt foci were grouped into small (1–3 crypts), medium (4–6 crypts) or large (7–9 crypts). Colon tissue deviating from normal morphology at gross examination was embedded in paraffin wax, sectioned (4–6 μm), and stained with haematoxylin and eosin for microscopic examination.

Statistics

One-way analysis of variance was used to analyse body weight, weight gain, and food consumption. A test for normal distribution (Shapiro-Wilk) of aberrant crypt foci data was carried out, and one-way analysis of variance was used to determine the effect of treatment on the development of aberrant crypt foci. Duncan's multiple range test was used for pairwise comparisons. A probability of $\leq 5\%$ was considered significant. All statistical calculations were carried out using SAS release 6.04.

Results

Seven mice died of peritonitis after the first dose with DMH due to an increase in pH in the DMH solution. This was corrected before the second injection. Because of these accidental deaths no DMH mice were killed after 4 weeks.

Body weight and food consumption

Apart from the observed accidental deaths in the DMH animals, no treatment-related signs of adverse effects in the clinical appearance of the animals were observed during the experiment.

The data revealed an initially significant relative decrease in body weight in the PhIP, DMH, and IQ animals, the IQ animals having the lowest decrease. The average body weight in the PhIP and IQ animals remained low compared with that of the controls (from 5–14% reduction) during the dosing period. However, no consistently significant changes in body weight gain were recorded from week 1 of the dosing period onwards.

The figures for food intake showed an initially significant decrease in the DMH-dosed animals, followed by a relative increase over the next three weeks. The data for the animals fed either PhIP or IQ showed that there was a significant decrease in food consumption from the initiation of the dosing period onwards.

Aberrant crypt foci

Aberrant crypt foci were not observed in the colon of control mice (group I). In animals killed after four weeks only the mice dosed with IQ and PhIP showed aberrant crypt foci. A much higher number of aberrant crypt foci were found in the IQ mice (31.8 ± 5.2) than in the PhIP mice (0.5 ± 0.3) (Table 2). Ten weeks after the first dose, aberrant crypt foci were found in all dosed groups. The mice dosed with IQ had both significantly more small ($P \leq 0.001$) and total aberrant crypt foci ($P \leq 0.001$) than the other dosed groups. In the AOM- and DMH-induced mice a substantially higher percentage of medium or large-sized aberrant crypt foci were seen compared with the PhIP and IQ-induced mice (Table 2).

In the IQ-fed animals the aberrant crypts were seen primarily in the caecal part of the colon, whereas the aberrant crypt foci in the DMH animals were seen primarily in the mid-colon. The aberrant crypt foci induced by PhIP and AOM were

Table 2. Influence of different diets on number and distribution of ACF in mice according to number of crypts in focus

			ACF/colon (Mean (SE))(%)			
Weeks	No of rats	Group	Small*	Medium	Large	Total
4	4	II	0.5 (0.3) (100)	0.0 (0.0) (0)	0.0 (0.0) (0)	0.5 (0.3)
	4	III	31.8 (5.2) (99)	0.3 (0.3) (1)	0.0 (0.0) (0)	32.0 (5.4)
	4	IV	0.0 (0.0) (0)	0.0 (0.0) (0)	0.0 (0.0) (0)	0.0 (0.0)
10	10	II	3.7 (1.0) (100)^{x}	0.0 (0.0) (0)	0.0 (0.0) (0)	3.7 (1.0)^{x}
	10	III	71.2 (4.2) (99)^{y}	0.2 (0.1) (1)	0.0 (0.0) (0)	71.4 (4.1)^{y}
	10	IV	1.6 (0.9) (88)$_{x}$	0.1 (0.1) (6)	0.1 (0.1) (6)	1.8 (1.0)^{x}
	10	V	7.9 (2.4) (90)^{x}	0.9 (0.3) (10)	0.0 (0.0) (0)	8.7 (2.7)^{x}

* Small foci, 1–3 crypts; medium foci, 4–6 crypts; large foci, 7–9 crypts.

^{x,y} Groups not sharing a common superscript (x,y) differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

distributed more randomly in the colon. No tumours were detected in any of the animals in groups II-V.

Discussion

The animals tolerated the dietary exposure to PhIP and IQ. The relative decrease in body weight in the PhIP and IQ animals supports data from studies in which mice were fed dietary levels of 0.04% and 0.03% PhIP and IQ, respectively (Ohgaki *et al*, 1984; Esumi *et al*, 1989). As to the lower food intake in the PhIP animals, a similar effect was observed in another study at the Institute of Toxicology in which mice were fed a diet of PhIP (Mortensen, 1995, personal communication). Whether the cause of the lowered food intake is a consequence of a specific toxicological effect of PhIP and IQ, or reduced palatability of the test diets, has not been established.

It has been widely reported that aberrant crypt foci are markers for the development of colon cancer (Bird, 1995a). Aberrant crypt foci have been suggested to be biologically heterogeneous lesions where only a subpopulation persists and develops into cancer (Bird, 1995b).

The interpretation of the aberrant crypt foci as precursor lesions is difficult because neither of these chemicals has been reported as being carcinogenic in the colons of mice (Ohgaki *et al*, 1984; Esumi *et al*, 1989). This suggests that the aberrant crypt foci induced by these two chemicals do not represent early stage colon cancer.

One explanation might be that PhIP and IQ are only weakly carcinogenic to the colon compared with other organs, and therefore tumours arise in the liver, forestomach and lung in the case of IQ (Ohgaki *et al*, 1984), and in the lymphoid tissue in the case of PhIP (Esumi *et al*, 1989) long before they arise in the colon. Whether PhIP induces tumours in other organs in mice is not yet known, as only the data on lymphomas have been published, and a thorough histological examination of this carcinogenicity study in mice (Esumi *et al*, 1989) still remains to be reported. Importantly, the susceptibility to colon carcinogens in mice is dependent on strain (Diwan and Blackman, 1980; Deschner *et al*, 1983), and many of the carcinogenicity studies on heterocyclic amines (including IQ and PhIP) have been performed on the hybrid mouse CDF_1 [(BALB/cAnN × DBA/2N)F_1] in which the susceptibility might not be very strong.

Another explanation for the lack of concordance might be that the aberrant crypt foci induced in the caecal part (the herringbone structure) of the colon – the case with IQ – have no potential to develop into cancer. This theory is supported by the data from Tudek *et al* (1989) where aberrant crypts induced by IQ and benzo[*a*]pyrene, both of which are not carcinogenic to mouse colon, were more common in the caecal end of the colon.

The well known mice colon carcinogens AOM and DMH induced a substantially higher percentage of medium- or large-sized aberrant crypt foci than either PhIP or IQ (Table 2), supporting the concept that aberrant crypt foci with a higher crypt multiplicity represent increasing potential to develop into cancer (Zhang *et al*, 1992; Magnuson *et al*, 1993).

In the AOM group no aberrant crypt foci were detected after 4 weeks. This was unexpected, as McLellan and Bird (1988a,b) in two studies with the same species and sex of mice and only a single dose of 15 mg/kg body weight induced 2.5 ± 0.4 and 2.6 ± 0.93 aberrant crypt foci per colon, respectively, after 4 weeks.

Even though we used a species of mice which is moderately resistant to DMH (Deschner *et al*, 1983), our results with DMH after 10 weeks (8.7 ± 2.7 aberrant crypt foci/colon) are also relatively low. Lam and Zhang (1991), who treated CF1 mice (a sensitive strain) twice orally with DMH (20 mg/kg body weight), found 13–17 aberrant crypt foci per animal in three different experiments 21 days after the last treatment. The distribution of the aberrant crypts after DMH induction agrees with the data from Tudek *et al* (1989).

Even though abundant published findings are available on aberrant crypt foci as an early marker for colon cancer, some investigations have failed to show a correlation between aberrant crypt foci and colon cancer (Hardman *et al*, 1991; Carter *et al*, 1994; Thorup *et al*, 1994; Kristiansen *et al*, 1995). In the study by Carter *et al* (1994) using DMH-induced mice, the authors proposed that aberrant crypt foci have little, if any, malignant potential in mice. Likewise, Steele and Kelloff (1993), testing 42 chemopreventive agents, found that all of the false positive data (positive crypt/negative tumour) were derived from mouse tumour studies and the authors indicated that some species differences may exist.

In light of these observations, our conclusion is that if cooked food mutagens such as IQ or PhIP are to be used as initiators in the aberrant crypt foci test, the use of rats may be a better choice because: (1) in carcinogenicity studies with rats, the colon is the target organ for both IQ (Takayama *et al*, 1984) and PhIP (Ito *et al*, 1991), whereas the target organs

in mice have been liver, forestomach and lung for IQ (Ohgaki *et al*, 1984), and the lymphoid tissue for PhIP (Esumi *et al*, 1989); (2) in rats induction of aberrant crypt foci in the colon has been demonstrated after both IQ and PhIP administration (Tudek *et al*, 1989; Takahashi *et al*, 1991; Hasegawa *et al*, 1993); (3) the distribution of aberrant crypt foci in the colon of IQ or PhIP-dosed rats is comparable with that in rats given AOM or DMH, and correlates well with the distribution of tumours (Tudek *et al*, 1989; Hasegawa *et al*, 1993).

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PAPER V

ELSEVIER

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CANCER LETTERS

Refined carbohydrate enhancement of aberrant crypt foci (ACF) in rat colon induced by the food-borne carcinogen 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ)

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Abstract

The aberrant crypt foci (ACF) bioassay has been used extensively to study the early effects of different dietary components on the colonic mucosa of laboratory rodents. ACF are proposed to represent preneoplastic lesions of colon cancer. Compared to the normally used initiators 1,2-dimethylhydrazine dihydrochloride (DMH) and azoxymethane (AOM), the use of a diet-related colon cancer initiator, such as the heterocyclic amine 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) formed during meat cooking, would probably give a more relevant insight into diet-related colon carcinogenesis. In the present study it is shown that a feeding regimen with continuous low IQ doses (0.03% in the diet) throughout a study period of 10 weeks has a significant effect on the induction of ACF in the colon of male F344 rats. In addition, the study illustrates that the incidence of the IQ-induced ACF can be modulated by the amount of refined carbohydrates in the diet. Rats given a high sucrose/dextrin diet showed a significantly higher number of ACF compared to rats given a diet high in starches. The effect on tumor outcome will await the termination of a ongoing parallel study.

Keywords: Aberrant crypt foci; 2-Amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ); Refined carbohydrates; Colon; Rats

1. Introduction

The aberrant crypt foci (ACF) assay has been used extensively to study the early effects of different dietary components on the colonic mucosa of laboratory rats and mice [1]. The induction of ACF is believed to be indicative of a potential carcinogenic effect in the large bowel [2–5]. In most of the published animal experiments studying dietary components the two colon carcinogens, 1,2-dimethylhydrazine dihydrochloride (DMH) or azoxymethane (AOM), not normally found in the diet, have been used as initiators. If food-borne initiators in the human diet, such as the heterocyclic amines, formed during cooking of fish and meat [6] could be used in the ACF-assay, this test would be even more useful as a model in screening for dietary modulation of colon carcinogenesis. Recently we investigated the use of the two food-borne rodent carcinogens, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4-,5-

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b]pyridine (PhIP) as initiators in the ACF assay with mice fed continuous low doses of IQ or PhIP. Based on the observations in the study we came to the conclusion that if cooked food mutagens such as IQ or PhIP were to be used as initiators in the ACF test, the preferred rodent to use would be rat [7].

The present paper describes the effect of IQ as initiator in the ACF assay using male Fischer 344 rats. To test the possible dietary modulation on IQ-induced ACF, groups of rats were offered a diet high in refined carbohydrates, which is known to have a pronounced effect on ACF development [8–10].

2. Materials and methods

2.1. Animals and housing

Forty 3-week-old male rats (Mol:Fischer 344) were obtained from Møllegård Breeding Center (Ll. Skensved, Denmark). The animals were kept in stainless steel wire cages, two animals per cage. During the study the temperature was maintained at 22 ± 1°C and relative humidity at 55 ± 5%, air was changed 8–10 times/h, and fluorescent light was on from 2100 to 0900 h.

Table 1
Composition of diets

Ingredients	Group			
	I	II	III	IV
Na-caseinate	20	20	20	20
Carbohydrate mix[a]	67.3	0	67.3	0
Carbohydrate mix[b]	0	67.3	0	67.3
Soya bean oil (with vitamins A, D, and E)	4	4	4	4
Mineral mixture	3.3	3.3	3.3	3.3
Vitamins B and K, choline chloride, inositol and methionine	1.4	1.4	1.4	1.4
Cellulose	4	4	4	4
IQ	0	0	0.03	0.03

All data are percentages.
[a]Carbohydrate mix: 45% cornstarch, 45% potato starch, 5% sucrose, 5% dextrin.
[b]Carbohydrate mix: 5% cornstarch, 5% potato starch, 45% sucrose, 45% dextrin.

2.2. Chemical

IQ was purchased from Toronto Research Chemicals (Ontario, Canada).

2.3. Diets

The animals were offered a pellet diet without or with 0.03% IQ, prepared by Altromin GmbH u. Co. KG, Lage, Lippe, Germany. The composition of the different diets is given in Table 1. Animals in group I received the basic semisynthetic diet based on the formula given in [11]. In the group II diet the carbohydrate pool was reversed from 90% starches and 10% dextrin/sucrose to 10% and 90%, respectively. Groups III and IV received diets similar to groups I and II supplemented with 0.03% IQ. Analyses of IQ in the batch of pellets prepared were in accordance with the amount added.

2.4. Experimental design

The animals were randomly assigned to the four experimental groups (ten animals per group). The animals were fed the group I basic diet in the 1-week acclimatization period and then allocated to their respective diets (Table 1). Body weight and food consumption were measured weekly. The animals were sacrificed after 10 weeks on the test diets. Complete gross necropsy was performed on all the animals. The large intestine was cut longitudinally, rinsed in 0.9% NaCl solution, and divided into two pieces. The two colon segments were pinned on a cork slab and fixed at 4°C in 4% neutral buffered formaldehyde for 20 h. To visualize the ACF the colon segments were processed according to [8]. ACF were enumerated and grouped into small (1–3 crypts) and medium (4–6 crypts) foci.

2.5. Statistical analysis

The parametric one-way analysis of variance (General Linear Models Procedure) was used to analyze body weight, weight gain, and food consumption and Duncan's multiple range test was used for pairwise comparisons. To analyze number and distribution of ACF between groups the non-parametric Wilcoxon two-sample rank-sum test (NPAR1WAY Procedure) was used. A probability of ≤5% was considered sig-

Table 2
Influence of different diets on number of ACF in rats and distribution of ACF according to number of crypts in focus

Group	ACF/colon		
	Small[a]	Medium[b]	Total
I	0[c]	0[c]	0[c]
II	0[c]	0[c]	0[c]
III	5.3 ± 0.9 (88)[d]	0.7 ± 0.4 (12)[d]	6.0 ± 1.0[d]
IV	10.3 ± 1.9 (97)[e]	0.3 ± 0.2 (3)[c,d]	10.6 ± 1.9[e]

Values are means ± SE; numbers in parentheses represent percentage of total; effective number of rats = 10 for each group; number of weeks on each diet = 10.
[a]Small foci, 1–3 crypts.
[b]Medium foci, 4–6 crypts.
[c,d,e]Groups not sharing a common superscript differ significantly when tested pairwise in Wilcoxon rank-sum test ($P \leq 0.05$).

nificant. All statistical calculations were carried out according to SAS release 6.11.

3. Results

During the experiment, no treatment-related signs of adverse effect in clinical appearance of the animals were observed. A slight but statistically significant decrease in body weight was seen in the IQ-dosed animals from the sixth week of dosing irrespective the composition of the diet. A concomitant decrease in food consumption was recorded in the IQ-fed animals, statistically significant compared to either of the other groups, from the first week of dosing.

The total number and distribution of ACF are presented in Table 2. For the categories small and total the amount of IQ-induced ACF reached the level of significance compared to their respective controls. With respect to the category medium, significance was found between groups I and III. Further, it was seen that the refined carbohydrate diet (group IV) induced a statistically significantly higher number of small and total ACF compared to standard diet (group III).

4. Discussion

The animals adapted well to the dietary exposure to IQ, and no influence of the change in dietary carbohydrate pool was recorded in clinical appearance, body weights or food consumption. The slight relative decrease in body weight in the IQ-fed animals supports the data from Takayama et al. [12].

The present study shows that a feeding regimen with continuous low IQ doses throughout the study period has a significant effect on the induction of the number of small ACF as well as the total number of ACF in rat colon. As far as we know this is the first ACF study using this protocol with low continuous IQ doses, which is more similar to human exposure to food-borne heterocyclic amines. Three other ACF studies have been conducted with IQ, and all have used few, high doses of IQ. Tudek et al. [13] treated female SD rats with either 100 or 200 mg/kg IQ twice by gavage, 4 days apart, and scored after 3 weeks, and Tachino et al. [14] and Liew et al. [15] used a dosing scheme with oral gavage of 130 or 100 mg/kg IQ, respectively, on alternating days for 2 weeks and scoring after 12 or 13 weeks, respectively.

Moreover, the study illustrates that the incidence of IQ-induced ACF can be affected by dietary modulators such as refined carbohydrates. IQ-dosed animals given a diet high in refined carbohydrates had a statistically significantly higher number of small and total ACF compared to IQ-dosed animals given standard diet. The increase in total number was approximately two-fold. The difference was not reflected in the medium-size ACF. The modulation of ACF by refined carbohydrates is seemingly inducer-dependent. In a recent study [8] with Wistar rats, where DMH was used as both initiator and promoter (20 mg/kg weekly for 20 weeks), the same high refined carbohydrate diet increased the total number of ACF 2.7-fold after 10 weeks feeding. The highest increase was seen in number of small ACF, but the number of medium, large and extra-large ACF were increased as well. In contrast, when AOM was used solely as initiator (15 mg/kg weekly for 2 weeks) no statistically significant effect on the number of ACF after 10 weeks feeding on a high sucrose diet was seen [16].

The modulating effect of diets high in refined carbohydrates on development of ACF has been reported by others. In DMH-induced rats Caderni at al. [9] found that a high-sucrose diet caused a significant increase in the percentage of large ACF (3–6 crypts) and a reduction in percentage of small ACF (1–2 crypts) compared with a high-starch diet. Stamp et al. [10] have compared the effect of the refined sugars sucrose, glucose, and fructose on ACF in AOM-induced mice.

The results showed that oral gavage of sucrose and fructose led to a significant increase in number of ACF while glucose did not. In addition, case-control studies have indicated that refined sugar may be a risk factor for human colorectal cancer [17–21].

Our results concerning refined sugars have shown that when studying colon cancer modulating test substances it may be important to investigate more than just one class of colon cancer initiators. This is also illustrated by the two compounds chlorophyllin (CHL) and indole-3-carbinol (I3C). CHL is an inhibitor of PhIP-induced ACF [22] and IQ-induced colon cancer [23]. However, CHL neither decreases nor increases AOM-induced ACF [24], and when DMH is used as initiator CHL acts as a promoter of colon cancer [25]. Similarly, I3C is a inhibitor of PhIP-induced ACF [22], but enhances DMH-induced colon carcinogenesis [26]. So, in addition to being of relevance to the human situation, the introduction of heterocyclic amines in the ACF assay in that way may also provide new information about the mechanisms behind colon cancer modulation.

The mechanism behind IQ induction of ACF in the colon is not known. The major site of activation of IQ to active metabolites is the liver. IQ is activated to a mutagenic metabolite by microsomes [27] and is a colon, small intestine, liver, Zymbal gland, skin, and clitoral gland carcinogen in rats [12]. Unchanged IQ is present in the gut when given perorally to rats and here it has been shown to be converted to a direct-acting mutagen 7-hydroxy-2-amino-3,6-dihydro-3-methyl-7*H*-imidazo[4,5-*f*]quinoline-7-one (7-OHIQ) by the gut bacteria. 7-OHIQ has also been detected in feces of humans ingesting a high level of fried meat. The bacterial metabolism of IQ, which is influenced by the composition of the diet, has been proposed to play a role in the induction of DNA damage in the colonic mucosa [28]. However, 7-OHIQ was not carcinogenic in rats in a study by Weisburger et al. [29], and they concluded that it is not likely that the bacterial metabolite of IQ, 7-OHIQ, accounts for the colon cancer risk in individuals consuming IQ and related heterocyclic amines. More likely, the carcinogenic effect may depend on the formation of *N*-hydroxy-IQ, or *N*-hydroxy-*N*-acetyl-IQ activated locally by enzymes such as *N,O*-acetyltransferase [29]. Oral administration of IQ results in formation of colonic mucosa-specific formation of DNA adducts in male F344 rats. The formation has been shown to be caused most likely by the absorption of carcinogen through the lumen [30].

In conclusion, the present study showed that a feeding regimen with continuous low IQ doses throughout the study period has a significant effect on the induction of ACF in rat colon and that a diet high in refined carbohydrates enhances the IQ-induced ACF. The effect on tumor outcome will await the termination of an ongoing parallel study.

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PAPER VI

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CANCER LETTERS

The role of aberrant crypt foci induced by the two heterocyclic amines 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) in the development of colon cancer in mice

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Abstract

Aberrant crypt foci (ACF) have recently been identified as early putative preneoplastic lesions which appear in the colons of experimental animals treated with colon carcinogens. In a recent study the two heterocyclic amines, 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP) were shown to be able to induce ACF in the colon of mice after, respectively, 4 and 10 weeks of exposure. In spite of the induction of ACF in colon of mice, IQ and PhIP have not been found to have colon as target organ in carcinogenicity studies. Therefore, one may question that ACF induced by IQ and PhIP in mice represent early stages of colon cancer. In order to investigate the possible role of PhIP- and IQ-induced aberrant crypt foci in the development of colon cancer in mice, colons from mice participating in other IQ- and PhIP-studies of much longer duration were analyzed for ACF. The results of these studies showed that the number of ACF increased statistically significantly over time, and that the small ACF were predominant (95–100%) at all time-points. In conclusion, this finding suggests that the detection of a high number of ACF with low crypt multiplicity (1–3 AC/Focus) in mice colon after IQ- or PhIP-treatment is not indicative for the end-point colon cancer, and thus supports the hypothesis that only the presence of a high number of ACF with high crypt multiplicity is predictive for tumor outcome.

Keywords: Aberrant crypt foci; Heterocyclic amines; 2-Amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ); 2-Amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP); Mice

1. Introduction

Food-borne heterocyclic amines, such as 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), are formed during cooking of meat and fish. They are mutagenic to bacteria and cultured mammalian cells and are carcinogenic in laboratory animals [1].

Colon cancer is believed to develop in a step wise manner in which genetic alterations accumulate and eventually lead to overt cancer [2]. Very early (2–4 weeks) after treatment with colon carcinogens, distinct morphological lesions known as aberrant crypt foci (ACF) can be observed in unembedded colon tissue stained with Giemsa or methylene blue. The

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induction of ACF is believed to be one of the early steps in colon carcinogenesis and to be indicative of a potential carcinogenic effect in the large bowel [3–6]. High frequencies of ACF have been observed in the colonic mucosa of rats and mice treated with colon carcinogens and in humans with increased risk of developing colon cancer [7].

In a recent study the two heterocyclic amines, IQ and PhIP, have been tested for their ability to induce ACF in the colon of mice after 4 or 10 weeks of exposure [8]. The two model colon carcinogens 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) were also tested. ACF were induced by all test substances. IQ induced significantly more small ACF than the other groups, while AOM and DMH induced a higher percentage of ACF ≥ 4 AC/Foci compared to the PhIP- and IQ-treated mice.

Two other studies on IQ- and PhIP-induced ACF in mice have been reported [9,10]. Tudek et al. [9] observed a few ACF in a 3-week study after administration of two doses, 4 days apart, of IQ (total dose 400 mg/kg body weight) and PhIP (total dose 150 mg/kg body weight) to CF1 mice. In a study using *MIN/+* mice, Steffensen et al. [10] found that PhIP given in four weekly i.p injections of 50 mg/kg body weight increased the number of both ACF and tumours in colons of the *MIN/+* mice [10].

In spite of the induction of ACF in colon of mice, IQ and PhIP have not been found to have colon as target organ in carcinogenicity studies. PhIP is a mouse lymphomagen, while IQ induces liver, lung and forestomach tumors in mice [11,12]. Therefore, one may question that ACF induced by IQ and PhIP in mice represent early stages of colon cancer.

To shed further light on the possible role of PhIP- and IQ-induced aberrant crypt foci in the development of colon cancer in mice, colons from wild-type mice participating in a series of other IQ- and PhIP-studies of longer duration were analyzed for ACF (Table 1). The aim of these studies was not to study ACF formation, and hence they were not designed as regular ACF-studies. However, due to the long duration of the studies, an investigation of the colons for ACF formation possibly could give some information about the fate of the ACF detected after 4 and 10 weeks treatment.

The present paper summarizes the results obtained from scoring ACF in colons from female mice in three studies where IQ or PhIP were used as initiating agents [8,13,14].

2. Materials and methods

The experimental conditions are given in Table 1. The animals received pelleted diets Altromin 1314 with 0, 0.03% IQ, or 0.03% PhIP added (Altromin GmbH u., Co KG, Lage, Germany). The treatment period and duration of the studies appear from Table 1. After sacrifice the large intestine was examined microscopically. Before fixation, the large intestine was cut longitudinally and rinsed in 0.9% NaCl solution. The colon was then pinned on a cork slab and fixed in 4% neutral buffered formaldehyde. To visualize the ACF the colon segments were processed according to Kristiansen et al. [15]. The aberrant crypts were identified by their increased size, the thicker and more deeply stained epithelial lining, and the increased pericryptal zone compared with normal crypts [16]. An ACF may consist of one to several crypts and in all three studies the ACF were grouped into small (1–3 crypts), medium (4–6 crypts), large (7–9 crypts) or extra-large (≥10 crypts). The experimental conditions have earlier been described in detail [8,13,14].

Study nos. 1 and 2 included wild-type mice as well as transgenic ET-*pim*-1 male and female mice, and ACF were observed in all IQ- and PhIP-treated mice, wild-type as well as transgenic. As the short-

Table 1

Experimental conditions for the studies included in the present paper

Study no.	Mouse strain	Sex	Treatment	Treatment period (weeks)/ Duration (weeks)	References
1	C57BL/6ByA	F	±0.03% PhIP in the diet	31/31 or up to 78	[13,14]
2	C57BL/6ByA	F	±0.03% IQ in the diet	24/40	[13]
3	C57BL/6J	F	±0.03% PhIP or IQ in the diet	4 and 10	[8]

Table 2
Effect of IQ on ACF-formation in female C57BL/6J (Study 3) and C57BL/6ByA (Study 2) mice

Study no.	No. of mice	Duration (weeks)	ACF/Colon[a,b]				
			Small	Medium	Large	Extra-large	Total
3	4	4	31.7 ± 5.2	0.3 ± 0.3	0	0	32.0 ± 5.4
3	10	10	71.2 ± 4.2[c]**	0.2 ± 0.1	0	0	71.4 ± 4.1[c]**
2	18	40	120.7 ± 10.2[d]***	5.6 ± 1.1[c,d]***	0.1 ± 0.1	0.1 ± 0.1	126.5 ± 10.6[d]***

[a]Values are means ± SE.
[b]Small foci, 1-3 crypts; medium foci, 4-6 crypts; large foci, 7-9 crypts; extra-large, ≥10 crypts.
[c]Differs from 4 weeks treatment.
[d]Differs from 10 weeks treatment. Wilcoxon rank-sum test.

term study (study no. 3) solely was performed on wild-type female mice, only ACF-data from female wild-type mice from study nos. 1 and 2 were used together with data from study no. 3 to compare the ACF-incidences over time.

Wilcoxon two-sample rank-sum test was used to analyze the number and distribution of ACF. A probability of ≤ 5% was considered significant. All statistical calculations were carried out using SAS release 6.11.

3. Results

3.1. No acf were detected in control animals

The results for IQ-dosed animals are presented in Table 2. A significant increase in number of small, medium and total number of ACF were seen over time. At all time-points small ACF (AC: 1–3) accounted for almost all of the ACF (99, 99, and 95%, respectively). No tumors were found in the colons of mice with ACF, and only a few lung and liver tumors were found [13], which are in accordance with the literature [11].

In Fig. 1, the number of ACF found in PhIP-induced mice during the studies is illustrated. In study no. 1, in which mice were sacrificed at two scheduled time-points, the mice died during the study as well. All PhIP-induced mice had ACF in their colon. It appears from the illustration that the induction of ACF increased until the exposure stopped. The number of induced ACF was still high shortly after termination of the exposure, but then it decreased. After 64 weeks the mean number increased but the data were based on very few mice and the increase at the terminal sacrifice (four mice) had a rather high standard error (mean ± SE = 12.0 ± 8.1).

In Table 3 the results for PhIP are presented. In this table the PhIP-induced ACF data from study no. 1 are pooled. Even though the data from study no. 1 are obtained at different time-points they are compiled in the table, just to give an impression of what is happening with PhIP-induced ACF over time. A significant increase in number of small, medium and total number of ACF was seen over time. At all three time-points small ACF accounted for almost all of the ACF (100, 100, and 96%, respectively). No colonic tumors were found in any of the PhIP-induced mice in study no. 1, but the study confirmed that PhIP is a potent mouse lymphomagen [12] as 70% of the wild-type mice, females as well as males, developed lymphomas [14].

4. Discussion

The present experiments showed that IQ and PhIP are able to induce ACF in mice, and that the number of small, medium, and total ACF increased statistically significantly over time. IQ induced ACF in a much higher number than PhIP. Almost all ACF induced at all time-points were small ACF (95–100%). No tumors were induced in mouse colons by the two compounds. Likewise, other PhIP-studies performed with wild-type mice of strain Balb C (unpublished data) and transgenic *Apc1638N* mice [17] in our laboratory have shown an induction of ACF in PhIP-treated colons.

It has been demonstrated in the literature that ACF are markers for development of colon cancer [7], and ACF have been suggested to be biologically hetero-

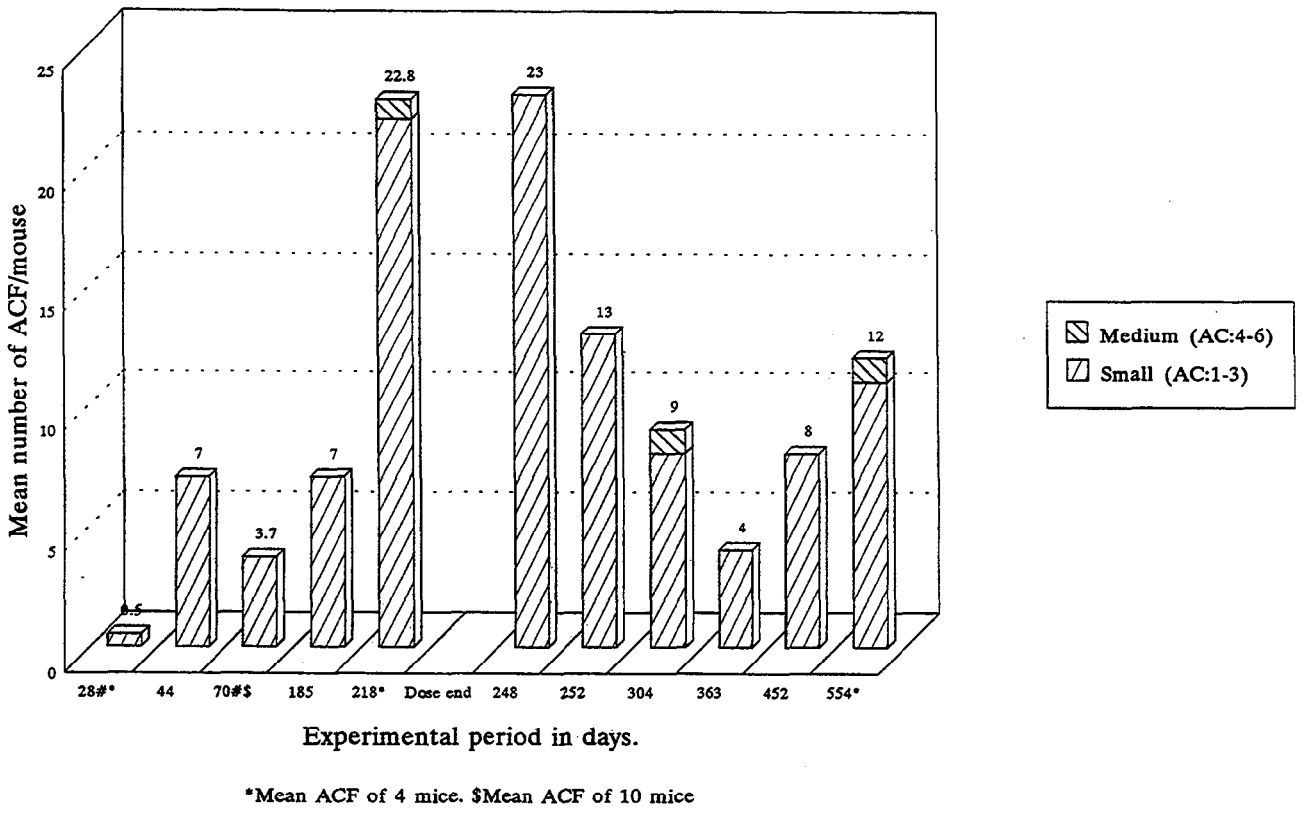

Fig. 1. Aberrant crypt foci in colons from C57BL/6ByA and C57BL/6J# female mice given 0.03% PhIP in the diet up to 218 days and followed until death.

geneous lesions where only a subpopulation persists and develops into cancer [18]. In addition, the presence of a high number of ACF with high crypt multiplicity has been hypothesized to be predictive for tumor outcome [19–21].

The mechanism behind IQ and PhIP induction of ACF in the colon is not known. Unchanged IQ is known to be present in the gut of mice after oral administration [22]. Activation of such IQ to a direct-acting genotoxin by the colonic microflora may thus lead to DNA damage in the colonic mucosa.

The lack of accordance between the induction of

Table 3

Effect of PhIP on ACF-formation in female C57BL/6J (Study 3) and C57BL/6ByA (Study 1) mice

Study no.	No. of mice	Duration (weeks)	ACF/Colon[a,b]				
			Small	Medium	Large	Extra-large	Total
3	4	4	0.5 ± 0.3	0	0	0	0.5 ± 0.3
3	10	10	3.7 ± 1.0[d]**	0	0	0	71.4 ± 1.0[d]**
2	15	Up to 78[c]	0.5 ± 2.8[e]***	0.5 ± 0.2[d,e]***	0	0	14.0 ± 2.9[e]***

[a]Values are means ± SE.
[b]Small foci, 1-3 crypts; medium foci, 4-6 crypts; large foci, 7-9 crypts; extra-large, ≥10 crypts.
[c]Pooled data, see Fig. 1.
[d]Differs from 4 weeks treatment.
[e]Differs from 10 weeks treatment. Wilcoxon rank-sum test.

ACF with PhIP and IQ and the development of tumors in carcinogenicity studies in mice [11,12], suggests that the ACF induced by these two chemicals do not represent an early stage of colon cancer. One explanation might be that PhIP and IQ are only weakly carcinogenic to the colon compared to other organs, and therefore tumors arise in the liver, forestomach and lung in the case of IQ [11], and in the lymphoid tissue in the case of PhIP [12] long before they develop in the colon. Another explanation might be that the present results support the hypothesis that only the presence of a high number of ACF with high crypt multiplicity is predictive for tumor outcome [19]–[21]. In both the IQ- and the PhIP-studies almost all ACF induced at all time-points were small ACF. Finally, the possibility that IQ and PhIP are only weak initiators of colon cancer which must be followed by a promotor for the induced ACF to develop into colonic tumors in mice needs to be investigated.

Another in vivo bioassay for detection of colon carcinogens is the nuclear aberration (NA) assay. The basis for this assay is that colon carcinogens, when given to mice, induce nuclear aberrations (i.e. micronuclei, karyorrhexis, and pyknosis (apoptotic bodies)) in colon crypts. In this NA assay, IQ in single doses of 200–800 mg/kg has been found to be a potent inducer of NA [23,24]. As nuclear aberrations are cell-lethal events, the induction of NA suggests that IQ, or its active metabolite, is capable of reaching the colonic epithelium and exerting nucleotoxic effects. Two other examples of non-colon carcinogens which both induce ACF and NA in mice colon are DMBA (7,12-dimethylbenz(*a*)anthracene) and benzo(*a*)pyrene [9,25].

Even though abundant literature is available concerning ACF as an early marker for colon cancer, some investigations have failed to show correlation between ACF and colon cancer in mice. In a study by Carter et al. [26] using DMH-induced mice, the authors concluded that the total number of ACF is not necessarily a valid indicator of the overall risk of overt tumor formation, and proposed that ACF have little if any malignant potential in the mouse. Likewise, Steele and Kelloff [27] testing 42 chemopreventive agents found that all of the false positive data (positive crypt/negative tumor) were derived from mouse tumor studies and the authors indicated that some species differences may exist.

In conclusion, the present results suggest, that the detection of a high number of aberrant crypt foci with low crypt multiplicity (1–3 AC/Focus) in mice colon after IQ- or PhIP-treatment is not indicative for the end-point colon cancer. In this way the results confirm the stated hypothesis that only the presence of a high number of ACF with high crypt multiplicity is predictive for tumor outcome [19]–[21]. Therefore, the present study illustrates that, to avoid false prediction of colon cancer potential in screening procedures, this hypothesis needs to be further investigated.

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