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Intestinal tumorigenesis in the *Apc*1638N mouse treated with aspirin and resistant starch for up to 5 months

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The *Apc*1638N mouse model, which carries a targeted mutant allele within the adenomatous polyposis (*Apc*) gene and develops intestinal tumours spontaneously, predominantly in the small bowel, was used to investigate the effects of two potential chemopreventive agents, aspirin and α-amylase resistant starch (RS). Heterozygous *Apc*+/*Apc*1638N mice were fed semi-purified diets rich in animal fat, animal proteins and sucrose and low in dietary fibre (Western style diets) from ~6 weeks up to 6 months of age. Two of the diets contained aspirin (300 mg/kg diet) and two RS (1:1 mixture of raw potato starch: Hylon VII at 200 g/kg diet) in a 2×2 factorial design. A fifth treatment group were fed a conventional rodent chow diet. The mice fed the Western style diets became almost three times as fat as the chow-fed mice but this did not affect tumour yield. Treatment with RS resulted in significantly more intestinal tumours whereas aspirin alone had no effect. However, there was a significant aspirin×RS interaction, which suggests that aspirin could prevent the small intestine tumour-enhancing effects of RS in this *Apc*-driven tumorigenesis model. The possibility that large amounts of purified forms of resistant starch may have adverse effects within the small bowel is a novel observation that requires further investigation since greater intakes of starchy foods (and of RS) are being encouraged as a public health measure in compensation for reduced dietary fat intake. However, it remains possible that any increased risk is restricted to carriers of germline mutations in *APC*.

Introduction

Mutations in the *APC* (adenomatous polyposis coli) tumour suppressor gene are early events in the aetiology of colorectal tumours, which arise both sporadically and from the transmission of germline mutations (1). The inherited disease, familial adenomatous polyposis (FAP), is characterized by the development of hundreds or thousands of adenomas in the colon and rectum; if untreated, FAP patients have a very high risk of developing colorectal carcinomas. In addition, FAP patients often develop extra-colonic lesions with peri-ampullary tumours being a particular problem both for diagnosis and surgical treatment (2,3). FAP has an autosomal dominant pattern of inheritance and is caused by germline mutations in the *APC* gene on chromosome 5q21 (4). In the majority of patients, the mutations lead to production of a truncated form of the APC protein (5). Whilst the function(s) of the APC protein have yet to be established, it appears to have a role in cell migration (6), cell adhesion and signalling (7) and in the regulation of both the cell cycle (8) and apoptosis (9,10). Mutations of the *APC* gene also occur in the majority of sporadic colorectal adenomas and carcinomas (11). In sporadic cases and in FAP-related tumours, the loss of activity of the *APC* gene product through mutation of both alleles appears to be an early step in tumorigenesis, probably preceding adenoma formation (12–14). This is followed by abnormalities in other tumour suppressor genes and oncogenes allowing the further growth of adenomas and the development of adenocarcinomas (1,11,15). *APC* appears to be the gate-keeper gene (16) so that integrity of this gene may be critical in defence against colorectal tumorigenesis.

Diet is an important environmental factor in the development of cancer (17,18) and diet may account for ~30% of cancers at all sites world wide (19). The evidence for the role of diet in the aetiology of colorectal cancer is particularly strong (20) with diets rich in fruits and vegetables and those low in fat being associated with low risk (18). A recent epidemiological study found that starch intake was the strongest dietary predictor of colorectal cancer incidence between countries with a highly significant negative relationship between starch intake and incidence of colorectal cancer (21). It was suggested that the active component was ‘resistant starch’ (RS), that is, the fraction of starch not absorbed in the small bowel. RS enters the large bowel where it is fermented, yielding short chain fatty acids including butyrate (22). Butyrate, a preferred substrate for normal human colonic epithelial cells (23), is a potent inducer of cellular differentiation (24), stimulates apoptosis (25,26) and may be responsible for the protective effects associated with diets rich in RS or non-starch polysaccharides (27,28).

Several epidemiological studies have shown that aspirin is associated with a reduced incidence of colorectal adenomas and adenocarcinomas in the general population (29–31). The extent of protection (up to 50% reduction in risk for regular aspirin users) is similar for both sexes and appears to be both dose and time of exposure dependent. This may be a general feature of non-steroidal anti-inflammatory drugs (NSAIDs) since studies have shown that sulindac suppresses polyp development in FAP patients and in cases with sporadic adenomatous polyps (32–36). In addition, the NSAIDs, piroxicam and sulindac, reduced the numbers of intestinal tumours in mice with inherited defects in *Apc* (37,38) whereas sulindac

Abbreviations: *Apc*, mouse adenomatous polyposis coli gene; COX-2, cyclooxygenase-2; FAP, familial adenomatous polyposis; NSAIDs, non-steroidal anti-inflammatory drugs; NSP, non-starch polysaccharides; RS, resistant starch.

Table I. Formulation (g/kg) of the experimental diets

	W1	RS	AS	RS + AS
Casein	280	280	280	280
Gelatine	20	20	20	20
Lard	250	250	250	250
Sucrose	150	150	149.7	149.7
Normal starch	250	0	250	250
Raw potato starch	0	125	0	125
Hylon VII[a]	0	125	0	125
Vitamins and minerals	50	50	50	50
Aspirin	0	0	0.3	0.3

W1, Western style; RS, resistant starch; AS, aspirin; RS + AS, resistant starch and aspirin.
[a]High amylose maize starch (National Starch and Chemical Company, Manchester, UK).

Table II. Starch and non-starch polysaccharides (g/100 g) contents of the diets[a]

Diet	RS	Non-starch polysaccharides
Western style	1.0	0.2
RS	12.9	0.2
AS	2.2	0.3
RS + AS	10.2	0.2
Chow	0	13.9

[a]Analyses performed by Dr H.N.Englyst (Dunn Clinical Nutrition Centre, Cambridge, UK).
Abbreviations as defined in Table I.

and aspirin reduced colonic tumour development in rodents treated with carcinogens (39,40). The mechanism of action of NSAIDs and specifically aspirin in reducing cancer risk are not known with certainty but inhibition of cyclo-oxygenase-2 (COX-2), which is expressed in tumours but not in normal colonic mucosa (41,42), is an attractive hypothesis (38,43).

Fodde *et al*. (44) produced a novel mouse model for FAP by introducing a chain termination mutation in the 15th exon of the mouse *Apc* gene (*Apc*1638N). This mutation was found to result in undetectable levels of the predicted truncated protein. In the first 6 months of life these mice spontaneously develop up to five or six intestinal adenomas and adenocarcinomas, mainly in the duodenum and jejunum (44,45). This model was used to assess the effects of dietary treatment with RS and aspirin, either alone or in combination, on the development of intestinal tumours.

Materials and methods

Experimental design

Groups of both male and female *Apc*1638N mice were fed one of four experimental diets (see Table I) or a standard rodent breeding diet (chow) from soon after weaning for up to 5 months. The four experimental diets were Western style, high in animal fat, animal proteins and sucrose and low in 'dietary fibre' and contained the treatments of RS and aspirin in a 2×2 factorial design. Animals were killed and tissues examined after ~1, 3 and 5 months of exposure to the treatments.

Mice and diets

Mice for the study were bred from F8 *Apc*1368N male mice and C57Bl6/J females. The young mice were weaned at 21–28 days of age. Both the breeding mice and the weaned young prior to the start of the study received a standard rodent breeding diet, SDS diet RM3 E (Special Diet Services, Witham, Essex, UK), hereafter referred to as chow. Mice were genotyped at a mean age of 35 days by conventional PCR-based procedures as previously described by Fodde *et al.* (44) and those carrying the *Apc* mutation were then assigned to one of the four experimental diets (Table I) or to the chow diet. The animals were offered 5 g of food per day and had free access to water. A control group of C57BL6/J mice received the Western style diet only. Analysis of the diets for polysaccharide content was carried out by Dr H.N.Englyst (Dunn Clinical Nutrition Centre, Cambridge, UK; Table II).

Tissue collection

The mice were killed and examined at three ages: M1 mice were aged between 65–74 days at death (mean 70 days) and were on the diets for 27–30 days (mean 29 days); M3 mice were aged 100–143 days at death (mean 122 days) and were on the diets for 53–92 days (mean 73 days); and M5 mice were aged 193–215 days at death (mean 204 days) and were on the diets for 153–164 days (mean 159 days). Approximately 20 mice from each treatment group were studied at M3 and M5, but only 4–5 mice at M1 (Table III).

One to five of the M5 cohort of mice from each treatment group (except the wild-type controls) became unwell (because of the development of intestinal tumours) and were killed early. Data from these mice are included in all results apart from those for body mass and fat content.

Tumour sampling

The mice were killed by CO_2 inhalation and the entire intestinal tract was removed, opened and flushed with saline. All macroscopically visible tumours were counted, the site was recorded and the tumours were taken for analysis by an observer (S.L.H.W.) who had no knowledge of the treatment group. Samples were fixed in 10% neutral buffered formalin and processed to paraffin wax. Sections were cut at 3 μm thick and stained with haematoxylin and eosin. Histological examination of the tumours was performed using standard criteria (46).

Body weight and fat content

The body weight of the animals was recorded after removal of the gastrointestinal tract. Mouse carcasses were freeze dried, cut into small pieces (<1 cm) and fat content was determined by exhaustive extraction with petroleum ether for 6 h (Soxhlet procedure). The defatted carcasses were ground finely and re-extracted with petroleum ether for a further 6 h.

Statistical analysis

Data were examined by analysis of variance using sex as a factor. Results for the chow diet were compared with those for the four Western style and then orthogonal contrasts were used to test for the effects of RS, aspirin and the interaction of RS + aspirin as *a priori* hypotheses using SPSS for Windows, Release 8.0.0. Separate analyses were made at each sampling time i.e. M1, M3 and M5.

Results

Experimental protocol

Direct analysis (Table II) demonstrated that the chow diet was rich in non-starch polysaccharides (NSP) in contrast to all four Western style diets, which contained only traces of NSP. The measured resistant starch in the RS and RS + AS (aspirin) diets was similar and numerically of the same order as the NSP content of the chow diet. There were no significant differences between the groups in duration of treatment or age at death when early deaths were included in the analysis (data not shown), which indicates that neither time of exposure to the treatments nor age was a confounding factor.

Distribution of tumours and tumour type

None of the control (C57BL6/J) mice developed tumours. The mean number of tumours per *Apc*1638N mouse increased with age in all treatment groups (Figure 1). Few tumours were seen in M1 mice (mean age 70 days). Most intestinal tumours were adenomas. No carcinomas were seen in either M1 mice or M3 mice (mean age 122 days). In the M5 mice (mean age 204 days) 13 out of the total of 187 intestinal tumours were invasive adenocarcinomas.

In all ages and in all groups the distribution of tumours between different sites was similar. No sex differences were observed. The duodenum was the most common site for tumours. Large intestinal tumours were rare: only one, a caecal adenoma, was observed in an M5 mouse. The distribution

Table III. Numbers and sex of mice in each treatment group

Treatment group	Age group M1	Age group M3	Age group M5	
			survivors	early deaths
1: W1	4 (2M, 2F)	20 (14M, 6F)	18 (13M, 5F)	2 (2M)
2: RS	5 (3M, 2F)	20 (7M, 13F)	16 (8M, 8F)	4 (4M)
3: AS	5 (4M, 1F)	20 (17M, 3F)	15 (7M, 8F)	5 (5M)
4: RS + AS	5 (2M, 3F)	20 (9M, 11F)	19 (8M,11F)	1 (1M)
5: Chow	4 (3M, 1F)	20 (13M, 7F)	19 (9M, 10F)	1 (1M)
C57BL6/J[a]	0	20 (10M, 10F)	20 (10M, 10F)	0

[a]Control mice with wild-type *APC*+/+ fed the Western diet.
Survivors, mice that remained healthy until sampling; early deaths, mice that appeared unwell and were therefore killed early.
Abbreviations as for Table I.

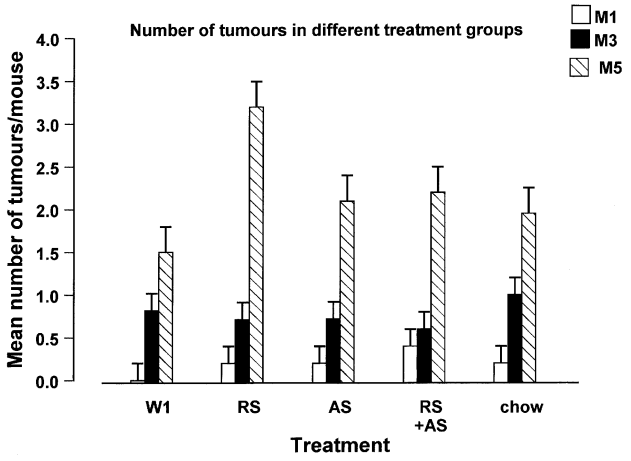

Fig. 1. Intestinal tumour yield for each treatment group of *Apc*1638N mice at each sampling time. W1, Western style diet; RS, resistant starch; AS, aspirin.

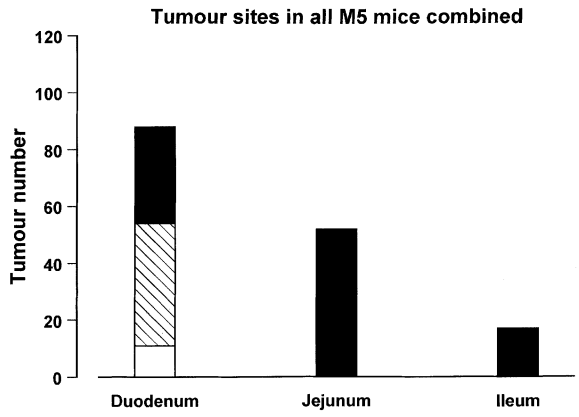

Fig. 2. Distribution of tumours within the small intestine of *APC*1638N mice after 5 months exposure to the test diets. For the duodenum: black bars, pylorus; hatched bars, peri-ampulla; and white bars, remainder of duodenum.

within the small bowel of tumours for all the M5 mice are shown in Figure 2. The majority of the adenomas showed the architectural features of tubular adenomas; a few were tubulovillous adenomas. No villous adenomas were seen. Most adenomas had moderate or severe (high grade) dysplasia; a few had mild (or low grade) dysplasia. The tumour architecture and degree of dysplasia were similar in all ages, sites and treatment groups. The proportion of adenocarcinomas was similar in all treatment groups (mean 0.16 per mouse).

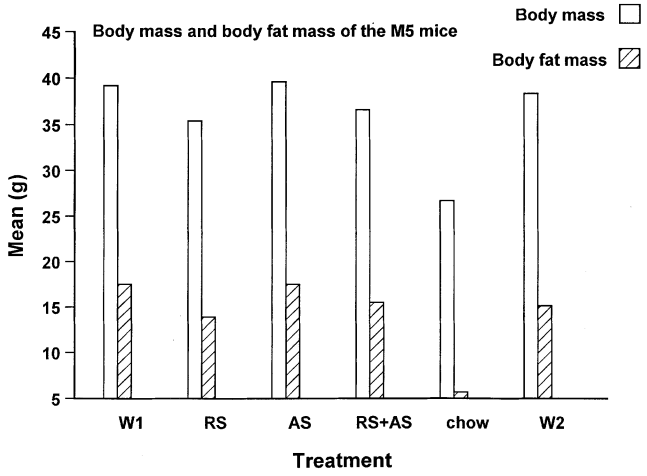

Fig. 3. Total body mass and body fat mass of mice fed Western style diets containing RS or aspirin (AS) or a conventional rodent chow diet (chow) for 5 months from weaning. W1 and W2 are the *Apc*1638N and wild-type (C57Bl6/J) mice, respectively, fed the basal Western style diet.

A few extra-intestinal abnormalities were found, including an occasional liver with mild steatosis. Many mice had enlarged spleens showing extramedullary haemopoiesis. Desmoid tumours and cutaneous cysts also occur in this model (64) but were not investigated in the present study.

Effects of treatments

No significant differences in the numbers of intestinal tumours between the treatment groups were seen in the two younger age groups (M1 and M3). At M5 there was no overall significant difference in the tumour burden between mice receiving the chow diet and those fed the four Western diets. However, within the latter diets, inclusion of RS in the diet increased the tumour number per mouse significantly ($P = 0.005$). The overall effect of aspirin was not significant, but mice receiving both aspirin and RS had fewer tumours than those receiving RS alone; the significant ($P = 0.02$) interaction between RS and aspirin suggests that aspirin blunted the tumourigenic effect of RS. There were no effects of treatment on tumour size.

Feeding the high fat, high sucrose Western diet for 5 months resulted in much heavier carcass weights than for animal offered chow ad libitum ($P < 0.001$). Much of this additional weight was caused by body fat, which was three times higher on average for Western compared with chow diets (Figure 3). Replacing conventional maize starch in the diet with RS lowered body fat content significantly ($P < 0.001$). The wild-

type (C57BL6/J) mice did not differ in body mass or body fat mass from the *Apc*1638N mice on the same Western diet.

Discussion

The spontaneous occurrence and intestinal distribution of tumours seen in this study are broadly similar to those reported in the original description of this mouse model (44) and confirm a more recent detailed analysis of the tumour pathology of *Apc*1638N mice (45).

The most unexpected finding of this study was that mice fed RS had an increased number of tumours compared with other animals. This runs counter to the strong epidemiological evidence that suggests a protective effect of starch-rich diets against colorectal cancer in humans (21). Current hypotheses suggest that the RS fraction is particularly important because it reaches the large bowel where it is fermented yielding butyrate and other short chain fatty acids. The greater production of acid endproducts depresses luminal pH resulting in a diminished production of cytotoxic and mutagenic secondary bile acids (47,62) and a lower risk pattern of cell proliferation within colonic crypts (47). *In vitro* studies have shown that butyrate slows the growth of colonic carcinoma cell lines (48,49) and increases apoptosis (25). A study of the effects of topically applied butyrate (using enemas) on chemically-induced colonic tumours in rats showed that butyrate reduced the number and size of tumours and produced a pattern of crypt cell proliferation that was more similar to the normal (28).

Earlier studies in rats with chemically induced colonic tumours yielded conflicting results on the influence of RS feeding on carcinogenesis (48,50–53). One study showed an increase in butyrate concentrations in colonic contents and faeces of rats given RS, but the tumour load of these animals was similar to that of the control animals (48). Another study demonstrated that rats given RS had more colonic tumours and more aberrant crypt foci (50). In contrast, other studies have reported a reduction in number and size of adenocarcinoma (52) or of aberrant crypt foci (51,52) with RS feeding, which was associated with high caecal butyrate content (51).

The dietary supply of potentially fermentable polysaccharide (RS and/or NSP) to the large bowel was of the same order (10–14 g/100 g diet) for both RS-containing and chow diets (Table II) and many fold-higher than for the basal Western style diet. In contrast to the results of human epidemiological studies (18,20), there was no evidence that either NSP or RS provided any protection against intestinal tumorigenesis in this mouse model.

The effects of RS on small intestinal tumours has not been examined previously in animals or in humans. The luminal environment of the small bowel is different from that in the large bowel, and would be expected to provide differing exposures to carcinogens and tumour-inhibiting substances. For example, fermentation does not occur to any significant extent within the healthy small intestine so that small bowel mucosal cells are unlikely to be exposed to extra butyrate or other protective substances, produced as a result of bacterial catabolism of resistant starch. The effects of RS on small bowel tumours are therefore difficult to predict, and extrapolation from information obtained by studying the colon may not be possible. Finally, the dose of RS used in this study (~11% by weight of the diet; Table II) was very much greater than probable human exposure with conventional foods. Estimates of RS intake in Europe are ~5–10 g/day; Europeans consuming very high starch diets might consume 40 g/day (54). It is possible that the forms of RS used in the present study (raw potato starch and high amylose maize starch) could have adverse effects on the small bowel when consumed in a purified form and in very high doses, but there is no evidence that populations consuming high starch diets are at enhanced risk of tumourigenesis [indeed the opposite seems to be the case (21)]. However, given the rarity of sporadic small bowel tumours, an adverse effect at this site may have gone unnoticed. Given the lack of effect of RS on tumour size, it may be inferred that the effect was on tumour initiation and perhaps a result of genomic damage that caused mutations in the second *Apc* allele (loss of heterozygosity for *Apc*).

Several large epidemiological studies have shown that long-term, frequent aspirin intake is associated with a 50–60% reduction in the risk of colorectal adenomas and carcinomas (29–31). Experimental administration of NSAIDs in people with FAP (32–35) and with sporadic adenomatous polyps (36), in animal models of *Apc*-driven tumorigenesis (37,38,55) and in animals with chemically induced colonic tumours (39,40) also supports the efficacy of aspirin and other NSAIDs in reducing the occurrence of colorectal tumours. *Min* mice, which have a nonsense mutation at position 850 in the *Apc* gene, develop abundant small intestinal tumours. Jacoby *et al*. (37) examined the effects of piroxicam, whereas Boolbol *et al*. (38) and Beazer-Barclay *et al*. (55) gave sulindac to *Min* mice; in both studies the mice receiving the drug had fewer intestinal tumours. Since the present study was completed, Barnes and Lee (63) have reported that inclusion of 250 or 500 p.p.m. aspirin in a semi-purified diet resulted in significantly reduced tumour multiplicity in the small intestine (but not the colon) of *Min* mice.

The mechanism of action of these drugs is probably multifactorial as there are several stages at which carcinogenesis may be arrested (56). Although there is uncertainty about which metabolic effects are important, there is strong evidence supporting the role of aspirin in modulating prostaglandin metabolism (57). Prostaglandin-independent mechanisms are also thought to be important, including increased apoptosis (34,58,59).

Perhaps surprisingly, we did not observe a significant difference in the numbers of tumours produced in animals receiving aspirin from those that did not. However, our mice did not receive aspirin until after weaning. The reduction in tumours seen with the administration of sulindac to *Min* mice was more marked with earlier administration of the drug (*in utero* rather than from weaning) (55). Whilst it remains possible that very early (intra-uterine or from birth) exposure to NSAIDs, including aspirin, may be more effective in suppressing tumour development, the recent report by Barnes and Lee (63) demonstrates that tumour suppression can be achieved when aspirin is administered to *Min* mice from 6 weeks of age, which is similar to the protocol used in the present study. The difference in response between the two models (*Min* and *Apc*1638N) may indicate that there is a mutation specific effect of aspirin. If this proved to be so, it could suggest that the responsiveness to NSAIDs of those with inherited Apc mutations will depend on the nature of the mutation. The animals that received RS and aspirin had fewer tumours than those given resistant starch alone. If RS increases small bowel tumorigenesis by a mechanism that circumvents the normal DNA damage sensing procedures or by down-regulating apoptosis (60), aspirin may activate backup cellular defence systems.

Obesity is a marked characteristic of populations consuming Western diets. There is evidence that obesity is linked with cancer risk, especially carcinomas of the endometrium and gall bladder (61). Feeding Western style diets to our mice resulted in a 3-fold increase in body fat content but no change in intestinal tumour burden compared with chow-fed animals. A similar lack of association between obesity and colon cancer risk has been reported from human epidemiological studies (20,18).

In conclusion, this study was designed to test the efficacy of two potential chemopreventative agents, RS and aspirin, in the *Apc*1638N mouse fed high fat Western style diets. There was no evidence that feeding a high fat Western style diet, which resulted in a 3-fold increase in body fat content, had any impact on intestinal tumorigenesis. To our surprise, high intakes of RS (provided by the isolated substances raw potato starch and Hylon VII) resulted in a significant increase in small bowel tumour number. Whereas aspirin alone had no effect on tumorigenesis, feeding aspirin together with RS appeared to suppress the tumour-enhancing effect of RS.

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