

## Effect of vegetable and carotenoid consumption on aberrant crypt multiplicity, a surrogate end-point marker for colorectal cancer in azoxymethane-induced rats

Philip J.Rijken<sup>1</sup>, Wim G.Timmer, Aart J.van de Kooij, Ingrid M.van Benschop, Sheila A.Wiseman, Michiel Meijers and Lilian B.M.Tijburg

Unilever Research Vlaardingen, PO Box 114, 3130 AC, Vlaardingen, The Netherlands

<sup>1</sup>To whom correspondence should be addressed  
Email: philip.rijken@unilever.com

**Epidemiological studies indicate that increased vegetable consumption reduces the risk of colorectal cancer mortality. In the present study we have investigated the effect of consumption of standard diets supplemented with freeze-dried vegetables (peas, spinach, sprouts and broccoli) and carotenoids (all-*trans*  $\beta$ -carotene and palm oil carotenoid extract) on surrogate end-point markers for colorectal cancer in an azoxymethane-induced rat model. Mean aberrant crypt multiplicity was reduced (19%) by the pea-supplemented diet only ( $P < 0.05$ ). The vegetable-induced effect was more apparent in aberrant crypt foci with higher multiplicity. Intervention with diets supplemented with peas, spinach, sprouts and a mix of all vegetables reduced the number of foci with  $>2$  aberrant crypts/focus by 37, 26, 23 and 26%, respectively ( $P < 0.05$ ). Even more pronounced effects were observed in foci with  $>3$  aberrant crypts/focus, with reductions of  $\sim 50\%$  in the pea and spinach intervention groups. All-*trans*  $\beta$ -carotene and palm oil-derived carotenoids, supplied at similar doses to those expected in the vegetable diets, inhibited ACM only marginally. Aberrant crypt foci formation in groups fed a sprout-supplemented diet prior to or following azoxymethane treatment was similar, indicating that this effect is due to inhibition of promotion rather than initiation of colorectal carcinogenesis. Vegetable and carotenoid consumption did not affect *in situ* proliferation of colonic crypt cells, as assessed by semi-automated image analysis of bromodeoxyuridine (BrdU)-positive nuclei. BrdU-negative nuclei of colonic crypt cells were reduced slightly in the combined vegetable groups, as compared with the control ( $P < 0.05$ ). These data: (i) are in line with epidemiological evidence regarding beneficial effects of vegetable consumption on colorectal carcinogenesis; (ii) indicate that consumption of several types of vegetables inhibits early post-initiation events in colorectal carcinogenesis; (iii) suggest that the vegetable-induced effect is more pronounced in advanced lesions; (iv) indicate that the carotenoid content of the vegetables ( $\alpha$ - and  $\beta$ -carotene) contributes only marginally to the vegetable-induced effects.**

### Introduction

Colorectal cancer ranks as the second most common cause of death by cancer in affluent societies (1–3). Epidemiological

**Abbreviations:** ACF, aberrant crypt foci; ACM, aberrant crypt multiplicity; AOM, azoxymethane; BrdU, bromodeoxyuridine; TEAC, Trolox equivalent antioxidant capacity.

evidence indicates that high consumption of fruit and vegetables is inversely associated with mortality by various cancers, including colorectal cancer (4–6). The combined effect of dietary and modifiable non-dietary factors, such as smoking, even suggests that non-hereditary colorectal cancer mortality is largely preventable (7).

In view of the strength of epidemiological evidence, it is surprising that experimental studies regarding the effect of fruit and vegetable consumption on colorectal cancer have received limited attention (8,9). However, intervention studies with purified compounds from fruit and vegetables suggest that these contribute to the chemoprevention of carcinogen-induced colorectal cancer (see for examples refs 10–15). The aim of the present study is to assess the potential protective properties of several vegetables and two carotenoid formulations with respect to the early stages of colorectal carcinogenesis. For this purpose, two surrogate end-point markers which have previously been correlated with colorectal cancer have been investigated.

One of the best characterized markers for colorectal cancer in carcinogen-induced animal models is aberrant crypt multiplicity (ACM). Aberrant crypts arise during the early stages of azoxymethane (AOM)-induced colorectal cancer, as a consequence of extensive methylation of DNA bases beyond physiological levels and subsequent accumulation of somatic mutations (16). In more advanced stages of carcinogenesis, aberrant crypt foci (ACF) with multiple aberrant crypts are formed, referred to as ACM. ACM correlate particularly well with the incidence of colorectal adenomas and carcinomas in AOM-induced rat models (17–20).

Important players in the multifactorial process of colonic epithelial maintenance and aberrant crypt formation include cell proliferation, apoptosis and necrosis. However, based on previous studies, it must be concluded that these markers for colorectal cancer are questionable diagnostic markers for tumour development, especially when assessed separately (25). We analysed the total number of cells in aberrant crypts *in situ* by analysis of both bromodeoxyuridine (BrdU)-positive and BrdU-negative nuclei in colonic crypts. These parameters yield information about the number of cells being either in S phase or in other phases of the cell cycle, respectively.

Since carotenoids may be among the active chemopreventive agents in vegetables (10–15), we attempted to assess the relative contribution of particular carotenoids to the vegetable-induced effects by testing these isolated compounds in doses similar to those present in vegetable-supplemented diets.

To obtain clues as to whether the intervention diets act on modulation of AOM-induced initiation or promotion events, one of the vegetable diets was fed prior to as well as following AOM treatment in two separate intervention groups.

### Material and methods

#### Animals

A total of 150 male Mol:SPRD Sprague-Dawley rats ( $300 \pm 20$  g) of 8 weeks of age were purchased from Møllegaard (Skemsved, Denmark) to be

**Table I.** Composition of the diets supplemented with freeze-dried vegetables and carotenoids (g/1000 kJ)

Group	Control	Spinach	Broccoli	Sprouts	Peas	Mix	$\beta$ -Carotene	$\alpha,\beta$ -Carotene
Corn starch	33.06	31.29	31.29	31.29	31.29	31.29	33.06	33.06
Ca casein	9.92 (15.1 en%)	7.85	7.85	7.85	7.85	7.85	9.62	9.62
Fat mix	9.46 (35.0 en%)	9.46	9.46	9.46	9.46	9.46	9.46	9.46
Solka Floc	3.43	1.96	1.96	1.96	1.96	1.96	3.43	3.43
Mineral	2.40	2.40	2.40	2.40	2.40	2.40	2.40	2.40
Vitamin	0.69	0.69	0.69	0.69	0.69	0.69	0.69	0.69
L-Cystine hydrochloride	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
Choline bitartrate	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Spinach		5.90					1.47	
Broccoli			5.90				1.47	
Sprouts				5.90			1.47	
Peas					5.90		1.47	
$\beta$ -Carotene							0.003	
$\alpha/\beta$ -Carotene								0.003

The energy density of the control and carotenoid groups was 58.95 g/1000 kJ; the other diets were 59.88 g/1000 kJ. The energy contribution of the vegetables was 5.4%. All diets were based on AIN 93M standard laboratory animal diet (27). The fat mix consisted of 23.75% cocoa butter, 57.15% palm oil, 17.23% safflower oil and 1.86% trisun.

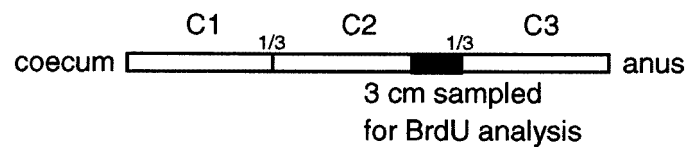
used as an AOM-induced animal model for colorectal cancer. Rats were allocated according to body weight in nine separate groups and received the diets depicted in Table I, except for one group (i.e. sprouts-delayed), which was temporarily kept on the control diet. The remaining animals (15), originally intended as spares for animals lost during AOM treatment or transport, were killed at the end of the study and used for method development purposes. Following 2 weeks of dietary intervention, rats were injected twice i.p. with AOM in saline (15 mg/kg body wt) within a 3 day interval. After an additional 2 weeks, the vegetable intervention diet in the sprouts-delayed group was started to allow discrimination between initiation and promotion events. To limit the size of the study, this aspect was considered for the sprouts group only. A saline control group was omitted, since it has been well documented that these animals do not develop aberrant crypts within the time frame of the study. One group of animals was injected with AOM, but received control diet throughout the study, to serve as a negative control for all dietary supplements. Prior to the intervention, one animal (group 3) died of unknown causes and one (group 8) showed signs of illness. These animals were removed and replaced by spares. At the end of the dietary intervention (14 weeks), rats were injected with BrdU i.p. (100 mg/kg body wt) and killed by decapitation after precisely 1 h.

#### Diets

Diets were based on the AIN-93M standard laboratory rodent diet and were corrected with corn starch and Ca casein to contain similar amounts of carbohydrate and protein. The average energy density was similar for all diets. Diets were adjusted for differences in insoluble fibre content due to freeze-dried vegetable supplementation with the relevant amount of Solka Floc (Table I). The standard control diet contained 40 mg/kg diet vitamin E. The other diets were supplemented with 10% (w/w) of one of the following lyophilized vegetables: peas, spinach, broccoli, Brussels sprouts or a mix of the four vegetables (2.5% w/w of each vegetable). Frozen raw vegetables were lyophilized to prevent a substantial reduction in energy density of the diets otherwise associated with the use of fresh vegetables with a high water content (80–90%). Two additional groups received diets supplemented with all-*trans*  $\beta$ -carotene (50 mg/kg diet; Roche, Basel, Switzerland) or the same dose of palm oil-derived carotenoids (50 mg/kg diet; Quest International, Naarden, The Netherlands). The palm oil carotenoids primarily consist of  $\alpha$ - and  $\beta$ -carotene in a ratio of 1:2. During the study, freeze-dried vegetables were stored under  $N_2$  at  $-20^\circ C$ . Fresh diet was prepared every 2 weeks and stored in the dark in black plastic bags at  $4^\circ C$ . Rats received fresh food every Monday, Wednesday and Friday. Fresh drinking water (*ad libitum*) was given twice a week and all diets were fed *ad libitum* throughout the study. Meals were removed ~2 h prior to killing.

#### Antioxidant activity of freeze-dried vegetable preparations

Antioxidant activity was determined by Trolox equivalent antioxidant capacity (TEAC) assay, as described by Miller *et al.* (22), to rule out loss of antioxidant activity due to preparation of the vegetables. For this purpose, accurately weighed samples of the frozen and thawed (wet) or the freeze-dried vegetables were added to boiling milli-Q water (5 times the wet vegetable weight). Vegetables were boiled for 5 min and carefully homogenized using a hand



**Fig. 1.** Sample area for BrdU analysis. BrdU staining and measurement was performed on the 3 cm C2 part of the colon (indicated in black).

blender. After cooling, samples were centrifuged at 15 000 g for 15 min and the supernatant was transferred to plastic tubes and stored at  $-70^\circ C$  until TEAC analysis. Alternative extraction methods have also been tested and these yielded similar results (data not shown).

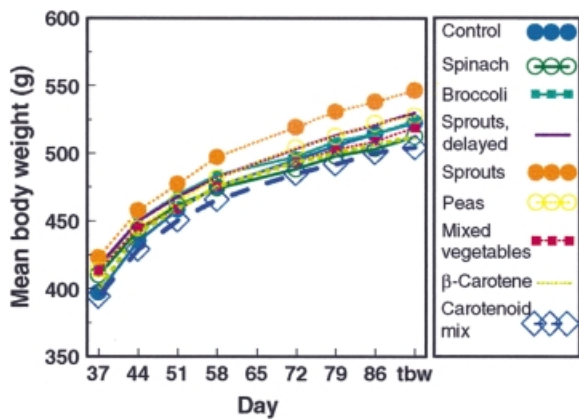
#### In situ assessment of ACM

At autopsy, the large intestine (colon + rectum) was removed and placed on cork in order to sample 3 cm of the middle colon (C2, Figure 1) for the assessment of BrdU-positive cells. To ensure that samples were taken from comparable regions of the large intestine, an adjustable elastic tape marked for three segments was used. The remaining parts of the large intestine were opened longitudinally and pinned flat on cork (mucosa side up) stretching the tissue laterally. After removal of the intestinal contents and cleaning of the surface by rinsing and brushing with phosphate-buffered saline (PBS), pH 7, both intestinal parts were fixed in 4% neutral phosphate-buffered formaldehyde. To visualize ACF, the colon was stained with 0.3% methylene blue for 0.5 min and washed in water for 1 min. AOM treatment induced aberrant crypt formation, reaching a level of 0.8–3.1 foci with  $\geq 3$  aberrant crypts/cm<sup>2</sup> within 14 weeks. Aberrant crypts are 2–3 times larger than normal crypts and characterized by a thicker and more deeply stained epithelial lining and an increased pericryptal zone which is elevated above the surrounding mucosa. A transparent grid, which segments the intestinal parts in fields of 2.5 × 5 mm, was placed over the intestine. The number of ACF and the multiplicity of each ACF was assessed in each field and ACM was analysed. Counting of ACF was carried out at 40× magnification, using a Zeiss stereomicroscope with a fibre optic light source.

#### In situ assessment of BrdU-positive and BrdU-negative cells

BrdU staining and measurement was performed on the 3 cm C2 part of the colon (Figure 1), cut open longitudinally, washed with PBS, pinned flat on cork (mucosa side up) and fixed in 10% neutral phosphate-buffered formaldehyde for 24 h. After fixation the sample was trimmed into four or five transversely cut sections of ~4 × 3 mm each. The samples were processed between biopsy pads in a Shandon Hypercenter XP Tissue Processor and all four or five samples were blocked in paraplast. For each animal/colon several 3.5  $\mu m$  paraplast sections were cut at intervals of 100  $\mu m$ .

In these sections the incorporated BrdU in the nuclei was visualized after pretreatment with hydrogen peroxide and subsequent incubation with normal goat serum, anti-BrdU (Becton-Dickinson, Leiden, The Netherlands), anti-mouse-IgG/biotin (Sigma, Zwijndrecht, The Netherlands) or extravidin/peroxidase (Sigma) and staining with diaminobenzidine (Sigma). BrdU-negative



**Fig. 2.** Mean body weight gain of dietary intervention groups. The average increase in body weight of the various groups was monitored from day 37 until death and was similar in all groups. The animals had no apparent preference for any of the diets. Terminal body weights (tbw) ranged from 504 to 546 g.

nuclei were stained with Groat's iron haematoxylin. A total of 20 crypts/animal were randomly selected for morphometry. The assessment of BrdU-positive and BrdU-negative nuclei was performed on colonic tissue from seven animals, which were randomly selected from each intervention group.

Morphometry was performed by means of a Zeiss IBAS Image Analysis Computer. In each selected crypt, total crypt depth, total crypt area, total surface area of all BrdU-positive material in the whole crypt (data not shown), total number of BrdU-positive nuclei and the total number of BrdU-negative nuclei were assessed.

#### Statistics

Data are presented as means  $\pm$  SEM of 15 animals/group, unless indicated otherwise. Statistics were performed with the SAS statistical program release 6.10. Data were analysed by means of ANOVA. Comparisons of the supplemented groups with the control were evaluated by ANOVA and Dunnett's one-tailed *t*-test. The level of significance was preset at  $P < 0.05$ .

## Results

### Food consumption and body weight gain

Composition of the diets was such that energy density and fat content were similar for all groups. Differences in carbohydrate content due to vegetable supplementation (17–50% w/w in the freeze-dried vegetable preparations) were corrected by reducing the amount of corn starch in the vegetable-supplemented groups with the average carbohydrate content (2.9% of total diet or 1.8 g/1000 kJ). In addition, the insoluble fibre content of the vegetable-supplemented diets was analysed (21–29%) and this was compensated for by adding an amount of Solka Floc (Table I). Food consumption by rats in the various intervention groups remained constant throughout the experimental period of 14 weeks (data not shown). In line with this observation, average body weight gain of all groups was not significantly different by the end of the study and resulted in terminal body weights ranging from 504 to 546 g (Figure 2). The animals had no apparent preference for any of the diets.

To exclude the possibility that the freeze-drying process had a detrimental effect on the antioxidant activity of the vegetables, total antioxidant activities in extracts from frozen (fresh) samples and from freeze-dried samples were determined by TEAC assay (Materials and methods). The results have been corrected for the increased extract volume due to the amount of water present in the original wet samples. Data are presented as mmol/l relative to Trolox per gram dry vegetable weight (Table II).

These data show that freeze-drying does not affect total antioxidant activity in the vegetable preparations used for

**Table II.** Effect of freeze-drying on TEAC values of several vegetables

	Frozen extract	Freeze-dried extract	Water (%)
Broccoli	0.146	0.145	85.4
Spinach	0.232	0.244	91.5
Peas	0.060	0.064	80.7
Sprouts	0.202	0.188	86.0

TEAC values of extracts from frozen wet vegetable extracts and extracts from freeze-dried vegetables were compared to check for loss of antioxidant activity by the freeze-drying process. Data are represented in mmol/l relative to Trolox (results are from a typical experiment,  $n = 5$ ).

supplementation of the diets. The TEAC value per gram dry vegetable weight was highest for spinach (0.244), followed by sprouts (0.188), broccoli (0.145) and peas (0.064).

### In situ assessment of ACM

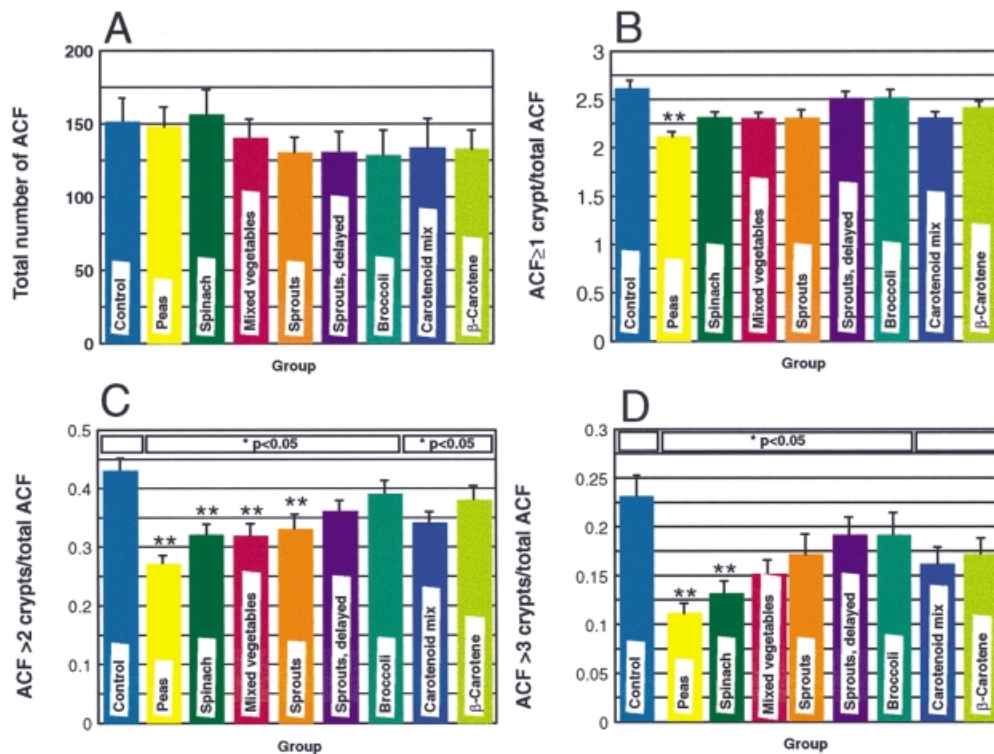
To determine the effect of vegetables or carotenoids on colorectal carcinogenesis, ACM was visualized by methylene blue staining. Subsequently, the total number of foci was determined and, with the exception of the area used for BrdU analyses, the complete colorectal area was analysed. The total number of aberrant crypts in all intervention groups was similar (Figure 3A). However, Figure 3B shows that the mean multiplicity per focus was significantly reduced in the pea-supplemented diet (19%,  $P < 0.05$ ), but not in the other groups. Interestingly, ACM with  $>2$  aberrant crypts/focus were significantly affected in additional intervention groups, leading to reductions in the peas, spinach, sprouts and vegetable mix groups of 37, 26, 23 and 26%, respectively ( $P < 0.05$ ; Figure 3C). The vegetable-induced decrease in ACM was even more pronounced in foci with a multiplicity  $>3$  ( $P < 0.05$ ; Figure 3D) in the pea- and spinach-supplemented groups. The other groups did not reach statistical significance here, which may be due to the smaller number of foci with high multiplicity, which results in higher variation coefficients.

Statistical evaluation of all vegetable intervention data combined suggests that vegetable consumption significantly reduces the formation of foci with multiple aberrant crypts ( $P < 0.001$ ). Supplementation with purified all-*trans*  $\beta$ -carotene or a palm oil carotenoid mix, however, did not significantly affect ACM, despite the fact that the doses were similar to the vegetable contents with respect to these carotenoids. Statistical analysis of the two carotenoid groups combined shows that the carotenoid-enriched diets affect ACM only marginally, as compared with the control ( $P < 0.05$ ).

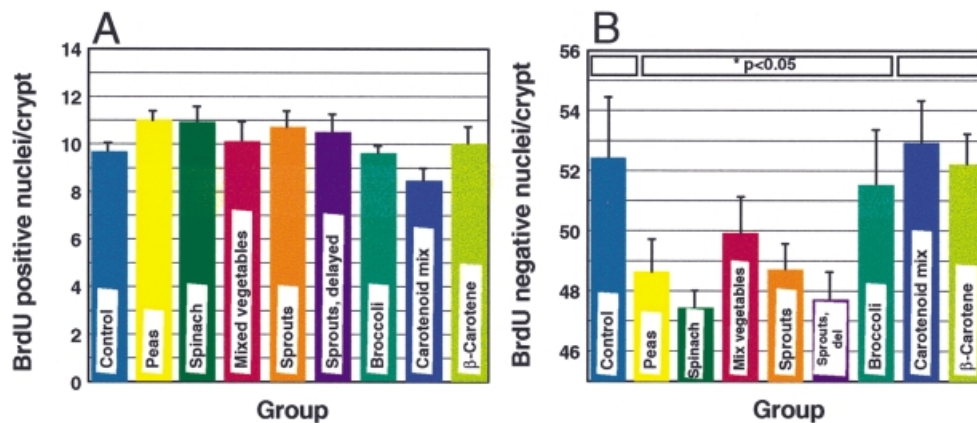
The difference between the two sprouts groups, either administered prior to or following initiation, was not significant (Figure 3B–D), indicating that sprout supplementation affected the post-initiation phase of colorectal carcinogenesis in this model. To limit the size of the study this latter aspect was tested in one group only.

### In situ assessment of BrdU-positive and BrdU-negative cells

To further investigate the mechanism involved in the vegetable-induced reduction in ACM, the fraction of colonic crypt cells in S phase was analysed by assessment of *in situ* BrdU-positive nuclei. Semi-quantitative analysis of BrdU-positive nuclei indicates that DNA replication was not significantly affected by any of the vegetable or carotenoid interventions (Figure 4A). A similar result was obtained when BrdU-positive nuclei were corrected for the total number of nuclei (not shown). However, analysis of BrdU-negative nuclei (Figure



**Fig. 3.** *In situ* assessment of the effect of vegetable and carotenoid supplementation on ACM. ACM were visualized by methylene blue staining and were determined as described (Materials and methods). The total number of ACF was determined in segments of 2.5×5 mm and, with the exception of the area used for BrdU analyses, the complete colorectal area was analysed. The mean multiplicity per focus was calculated (A), as well as the number of foci with >1 aberrant crypts/focus (B), the number of foci with >2 aberrant crypts/focus (C) and the number of foci with >3 aberrant crypts/focus (D). Statistical significance ( $P < 0.05$ ) was determined by ANOVA and is indicated by \*\*. Differences in ACM of the combined vegetable and the combined carotenoid groups reach statistical significance ( $P < 0.05$ ) as compared with the control group, as indicated by \* at the top of the figure.



**Fig. 4.** *In situ* assessment of the effect of vegetable and carotenoid consumption on the number of BrdU-positive and BrdU-negative nuclei in the colonic crypt epithelium. The number of BrdU-positive nuclei/crypt (A) and BrdU-negative nuclei/crypt (B) was analysed by semi-automated image analysis of the colonic epithelium. Statistical significance ( $P < 0.05$ ) was determined by ANOVA. BrdU-negative nuclei/crypt is significantly reduced in the combined vegetable groups, as compared with the control group ( $P < 0.05$ ). This is indicated by \* at the top of the figure.

4B) indicates that a marginal, but significant, loss of colonic crypt cells occurs in the vegetable-supplemented groups, as compared with the control ( $P < 0.05$ ) or as compared with the combined carotenoid groups ( $P < 0.005$ ). In line with this observation is that the total crypt area was significantly reduced in the combined vegetable groups, as compared with the control group ( $P < 0.05$ ; data not shown). These data indicate that one of the factors involved in the vegetable-induced reduction in ACM may be an increased loss of cells, but in view of the marginal effect it seems unlikely that this was a major factor.

### Discussion

The present study was designed to assess whether vegetable or carotenoid consumption affects colorectal cancer pathogenesis in a carcinogen-induced animal model. The study outcome provides experimental support for the intriguing findings consistently being reported in epidemiological case-control and cohort studies (4–7).

Previous reports about the effect of vegetable consumption on colorectal carcinogenesis in chemically induced or genetic models have indicated either no effect, significant protection,

a trend for protection or even increased risk due to fruit and vegetable consumption. The diets used for these studies were typically prepared for human consumption (8,9). To limit confounding issues, we have supplemented the diets with lyophilized vegetables or carotenoids only and corrected for differences between the intervention diets in energy density and fibre, protein, carbohydrate and fat content. Vegetables were not further processed (e.g. cooked), partly to avoid introduction of additional differences in the diets due to processing. In addition, the cooking process is highly variable among people, some people eating the vegetables raw or blanched whereas others cook them extensively. Based on these considerations, it was decided not to include the effect of breakdown products of food constituents in the design of this particular study. All diets were based on the AIN-93 standard laboratory diet (Materials and methods).

The data presented show that vegetable consumption is inversely associated with ACM, a surrogate end-point marker for colorectal cancer. Peas, spinach, sprouts and a vegetable mix appear more effective than broccoli, palm oil carotenoids and all-*trans*  $\beta$ -carotene, which show marginal effects. Although the effects of the intervention diets may have been partly masked by the composition of the standard control diet, which contains vitamin E, this suggests that different types of vegetables differentially affect colorectal carcinogenesis.

Most likely, the vegetable-induced effects on ACM cannot be attributed to the  $\alpha$ - and  $\beta$ -carotene present in the vegetables, considering the marginal effects in the carotenoid intervention groups on this marker. The carotenoid intake of the vegetable groups (0.1–1.5 mg/day for  $\beta$ -carotene) was similar to that of the carotenoid-supplemented groups (up to 1.5 mg/day). Although the bioavailability of carotenoids from the vegetables may be less than that from the supplemented diets, this may be compensated for by biological activity exerted by other carotenoids in the vegetables, besides  $\alpha$ - and  $\beta$ -carotene.

ACM may be considered a vector or surrogate end-point marker for colorectal cancer risk, since it has been demonstrated to be a consistent predictor of tumour outcome (17–19). In contrast, the total number of aberrant crypts as such is not a reliable quantitative predictor for the development of colorectal cancer (18,22), although in some studies a positive correlation was found (11). This may be due to the fact that foci with multiple aberrant crypts reflect a degree of aberrant behaviour of the colonic crypt epithelium found in more advanced stages of colorectal carcinogenesis (16,17).

The set-up of this study does not allow correlation with the disease end-point. When such correlations are pursued, they should preferably include information on tumour size and distribution and classification of the precise morphological and genetic characteristics of the tumour, clearly aspects beyond the scope of the current study.

ACM have been associated with colon neoplasia in humans as well, but the relation between these lesions and human colorectal cancer remains to be further investigated (18,24,26).

In a previous study with a carcinogen-induced model it was demonstrated that a 'low risk' diet with regard to fat, fibre and calcium significantly reduced DNA damage, as assessed by Comet assay in a dimethylhydrazine-induced rat model for colorectal cancer (21). DNA damage, however, did not correlate with ACM in this animal model. Despite lack of evidence for this, one explanation may be that carcinogen-induced models are not well-suited to study initiation events, due to the extreme dose of carcinogen used over a short time frame normally not

found in real life. The observations with the sprouts diet administered either before or after initiation with AOM support this hypothesis.

No effect on BrdU-positive nuclei was observed by any intervention regime, but the total number of BrdU-negative nuclei in colonic crypts was significantly reduced in the combined vegetable groups. A similar result was obtained when BrdU-positive nuclei were corrected for the total number of nuclei. In line with this observation, the total crypt area was significantly reduced in the combined vegetable groups, as compared with the control group ( $P < 0.039$ ; data not shown). This effect may be due to enhanced apoptosis or necrosis (25), although we have not been able to substantiate this hypothesis by *in situ* end-labelling of apoptotic DNA fragments in histopathological wax sections (ApopTag plus kit; Appligene-Oncor, France), due to insufficient signal obtained after labelling. Since the total number of cells was reduced in the vegetable-supplemented groups (as compared with the carotenoid groups,  $P < 0.05$ ), an alternative explanation is that the proportion of cells in S phase is increased in the vegetable-supplemented groups. However, the contribution of such processes to the vegetable-induced effect on ACM is questionable in view of the marginal effects.

A straightforward correlation of ACM with the antioxidant activity in crude vegetable extracts was not found. Peas had the lowest antioxidant activity, followed by broccoli, sprouts and spinach. The antioxidant potential as assessed by TEAC therefore most likely bears no relation to the observed effects on ACM.

## Acknowledgements

The authors would like to thank Richard Hambly for the apoptosis experiments and Jan Weststrate, Edward Haddeman, Wim Kloots, Koos van Wijk, Wim Tuijtel, Jan van Toor, Henk van Toor, Wil van Oort and Rinus Boers for critical discussions, expert assistance in the experimental phase of the study and careful preparation of the diets. Statistical expertise from Tom Wiersma was greatly appreciated.

## References

1. Cancer Research Campaign (1993) *Cancer Res. Campaign Factsheet*, **18**, 1–4.
2. SEER (1997) SEER Cancer Statistics Review. *J. Natl Cancer Inst.*, **89**, 416.
3. SEER (1997) SEER Cancer Statistics Review 1973–1994. *J. Natl Cancer Inst.*, **89**, 1093.
4. Block, G. (1992) A role for antioxidants in reducing cancer risk. *Nutr. Rev.*, **50**, 207–213.
5. Willet, W.C., Colditz, G.A. and Mueller, N.E. (1996) Strategies for minimizing cancer risk. *Scient. Am.*, **1996** (September), 58–63.
6. Ames, B.N., Gold, L.S. and Willet, W.C. (1995) The causes and prevention of cancer. *Proc. Natl Acad. Sci. USA*, **92**, 5258–5265.
7. Giovannucci, E. and Willet, W.C. (1994) Dietary factors and risk of colon cancer. *Ann. Med.*, **26**, 443–452.
8. Rijnkels, J.M., Hollanders, V.M., Woutersen, R.A., Koeman, J.H. and Alink, G.M. (1997) Interaction of dietary fat with a vegetables-fruit mixture on 1,2-dimethylhydrazine-induced colorectal cancer in rats. *Nutr. Cancer*, **27**, 261–266.
9. Kranen, H.J., van Iersel, P.W.C., Rijnkels, J.M., Beems, D.B., Alink, G.M. and van Kreijl, C.F. (1998) Effect of dietary fat and a vegetable-fruit mixture on the development of intestinal neoplasia in the *APC<sup>min</sup>* mouse. *Carcinogenesis*, **19**, 1597–1601.
10. Deschner, E.E., Ruperto, J.F., Wong, G.Y. and Newmark, H.L. (1991) Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenesis*, **12**, 1193–1196.
11. Alabaster, O., Tang, Z.C., Frost, A. and Shivapurkar, N. (1995) Effect of  $\beta$ -carotene and wheat bran fibre on colonic aberrant crypt and tumour formation in rats exposed to azoxymethane and high dietary fat. *Carcinogenesis*, **16**, 127–132.

12. Komaki,C., Okuno,M., Onogi,N., Morikawi,H., Kawamori,T., Tanaka,T., Mori,H. and Muto,Y. (1996) Synergistic suppressin of azoxymethane-induced foci of colonic aberrant crypts by the combination of  $\beta$ -carotene and perilla oil in rats. *Carcinogenesis*, **17**, 1897–1901.
13. Kawamori,T., Tanaka,T., Hirose,Y., Ohnishi,M. and Mori,H. (1996) Inhibitory effect of *d*-limonene on the development of colonic aberrant crypt foci induced by azoxymethane in F344 rats. *Carcinogenesis*, **17**, 369–372.
14. Deschner,E.E., Ruperto,J.F., Wong,G.Y. and Newmark,H.L. (1993) The effect of dietary quercetin and rutin on AOM-induced acute colonic epithelial abnormalities in mice fed a high fat diet. *Nutr. Cancer*, **20**, 199–204.
15. Wargovich,J.M., Chen,C.-D., Jimenez,A., Steele,V.E., Velasco,M., Stephens,L.C., Price,R., Gray,K. and Kellof,G.J. (1996) Aberrant crypts as a biomarker for colon cancer: evaluation of potential chemopreventive agents in the rat. *Cancer Epidemiol. Biomarkers Prev.*, **5**, 355–360.
16. Zaidi,N.H., Pretlow,T.P., O’Riordan,M.A., Dumenco,L.L., Allay,E. and Guron,S.L. (1995) Transgenic expression of human MGMT protects against azoxymethane-induced aberrant crypt foci and G to A mutations in the *K-ras* oncogene of mouse colon. *Carcinogenesis*, **16**, 451–456.
17. McLellan,E.A., Medline,A. and Bird,R.P. (1991) Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. *Cancer Res.*, **51**, 5270–5274.
18. Pretlow,T.P., O’Riordan,M.A., Pretlow,T.G. and Stellato,T.A. (1992) Aberrant crypts in human colonic mucosa: putative preneoplastic lesions. *J. Cell. Biochem.*, suppl. 16G, 55–62.
19. Magnuson,B.A., Carr,I. and Bird,R.P. (1993) Ability of aberrant crypt foci characteristics to predict tumor incidence in rats fed cholic acid. *Cancer Res.*, **53**, 4499–4504.
20. Shivapurkar,N., Tang,Z.C. and Alabaster,O. (1992) The effect of high-risk and low-risk diets on aberrant crypt and colonic tumor formation in Fischer-344 rats. *Carcinogenesis*, **13**, 887–890.
21. Hambly,R.J., Rumney,C.J., Cunnigham,M., Fletcher,J.M.E., Rijken,P.J. and Rowland,I.R. (1997) Influence of diets containing high and low risk factors for colon cancer on early stages of carcinogenesis in human flora-associated (HFA) rats. *Carcinogenesis*, **18**, 1535–1539.
22. Miller,N.J., Rice-Evans,C.A., Davies,M.J., Gopinathan,V. and Milner,A. (1993) A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.*, **84**, 407–412.
23. Hardman,W.E., Cameron,I.L., Heitman,D.W. and Contreras,E. (1991) Demonstration of the need for end point validation of putative biomarkers: failure of aberrant crypt foci to predict colon cancer incidence. *Cancer Res.*, **51**, 6388–6392.
24. Yamashita,N., Minamoto,T., Ochia,A., Onda,M. and Esumi,H. (1995) Frequent and characteristic *K-ras* activation and absence of p53 protein accumulation in aberrant crypt foci of the colon. *Gastroenterology*, **108**, 434–440.
25. Chang,W.-C.L., Chapkin,R.S. and Lupton,J.R. (1997) Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. *Carcinogenesis*, **18**, 721–730.
26. Roncucci,L., Medline,A. and Bruce,W.R. (1991) Classification of aberrant crypt foci and microadenomas in human colon. *Cancer Epidemiol. Biomarkers Prev.*, **1**, 57–60.
27. Reeves,P.G., Nielsen,F.H. and Fahey,G.C. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition *ad hoc* writing committee on the reformulation of the AIN-761A rodent diet. *J. Nutr.*, **123**, 1939–1951.

*Received November 19, 1998; revised September 1, 1999; accepted September 3, 1999*