Colonic fermentation of complex carbohydrates in patients with familial adenomatous polyposis

D M Bradburn, J C Mathers, A Gunn, J Burn, P D Chapman, I D A Johnston

Abstract

Decreased production of butyric acid by colonic carbohydrate fermentation may predispose to colonic carcinogenesis, with the implicit assumption that the decrease in faecal butyrate found predates the development of the tumour. The influence of the genetic predisposition to colonic tumours and the presence of colonic polyps on in vitro fermentation of carbohydrates was examined. Stool samples from 11 normal controls and 20 patients with familial adenomatous polyposis (FAP) were incubated anaerobically with a range of carbohydrates. Fermentation patterns were similar for glucose and raffinose. These sugars produced different short chain fatty acid (SCFA) patterns from the two polysaccharides, starch and arabinogalactan, which differed one from the other. The FAP gene carriers with polyps produced less butyrate than normal controls ($p < 0.005$) and gene carriers without polyps ($p < 0.05$). There were corresponding decreases in the molar ratios of butyrate. Gene carriers without polyps produced less absolute amounts of acetate than normal controls ($p < 0.05$) and slightly less total SCFAs ($p < 0.05$) but were otherwise not significantly different. The decreased production of butyrate noted by other workers may be secondary to the tumours rather than a contributory cause.

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Epidemiology has stressed the importance of diet in the development of colorectal cancer. Diets high in complex carbohydrates seem to be protective, whereas those high in fat may have adverse effects.^{1,2} Despite this epidemiological evidence, the importance of diet remains unproved and possible mechanisms have been sought. Burkitt originally suggested that the increased faecal bulk and decreased transit time associated with a diet rich in plant cell walls (dietary fibre) diluted carcinogens and lessened the contact time with the colonic mucosa.³ The realisation that dietary fibre is fermented in the colon to short chain fatty acids (SCFAs) has led to alternative hypotheses.⁴

The production of SCFAs is associated with a reduction in pH that has been proposed to be a protective factor through an, as yet, uncertain mechanism.⁵ Also, attention has focused on butyric acid, one of the SCFAs, which has antineoplastic properties in vitro.⁶ When added to malignant cell lines, butyrate is associated with the return of contact inhibition⁷ and the recovery of the morphological and functional characteristics associated with the parent cell.⁸ Decreased molar ratios of butyrate have been found in enema samples⁹ and in the faeces of patients with

colonic cancers and polyps.¹⁰ Decreased butyrate production was reported in similar patient groups.¹¹ It has therefore been hypothesised that dietary fibre is protective by virtue of its production of butyric acid.¹

For this hypothesis to be tenable, the reduction in butyrate must predate the development of tumours. We attempted to test this hypothesis with subjects with familial adenomatous polyposis (FAP). As subjects can be identified before polyps develop,¹² it is possible to separate changes that predate polyp growth from those that appear after polyps develop.

Patients and methods

SUBJECTS

Thirty one subjects were recruited from the Northern Region Polyposis Registry. Table 1 shows that risk analysis allowed the patients to be divided into three groups. All gene carriers with polyps had a colonoscopy as had seven of 10 gene carriers with no polyps. The remainder of the second group had rigid sigmoidoscopy. One of the controls had colonoscopy and eight had rigid sigmoidoscopy. Two controls (ages 9 and 11) were children of a departmental member and were not endoscoped. No patient had received any antibiotics in the six weeks before the study. All subjects were on a mixed western diet and no dietary restrictions were imposed.

With the estimates of interindividual variation and between group differences in butyrate molar ratios given by Weaver *et al*,⁹ it was calculated that 6-21 subjects in a group would provide an 80% power of detecting a true difference of the size reported by Weaver *et al*⁹ at the 5% level of significance.

SAMPLE COLLECTION AND PROCESSING

Faecal samples were collected in the patient's own homes and were stored anaerobically in sealed evacuated plastic bags at 4°C for up to six hours until homogenisation by a stomacher (model 400, Seward Medical) for five minutes. After pH measurement (Corning, model 245), duplicate 1 g portions were taken for SCFA analysis and frozen. A further 20 g sample was taken and stored anaerobically under CO₂ at 4°C overnight in preparation for in vitro fermentation.

IN VITRO BATCH CULTURE

Faeces (20 g) were homogenised with 125 ml of buffer¹³ at 37°C and filtered through a single layer of muslin. Five millilitres of filtered slurry and 10 ml of buffer with either no carbohydrate

Department of Surgery
D M Bradburn
I D A Johnston

Department of Biological
and Nutritional Sciences
J C Mathers

Department of Human
Genetics, University of
Newcastle upon Tyne,
Framlington Place,
Newcastle upon Tyne
J Burn
P D Chapman

Ashington Hospital,
Northumberland
A Gunn

Correspondence to:
Mr D M Bradburn,
Department of Surgery,
Medical School, Framlington
Place, Newcastle upon Tyne
NE2 4HH.

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TABLE I Risk of familial adenomatous polyposis (FAP) gene carriage¹² and ages of the study population

Group	No	Risk of gene carriage (%)	Mean age (range) (y)
Control	5 M 6 F	<1	25.6 (9-53)
FAP gene carriers with no polyps	3 M 7 F	>98	22.3 (7-53)
FAP gene carriers with polyps	7 M 3 F	100	19.9 (11-32)

TABLE II Analysis of variance model for testing the outlined experimental hypotheses

Source of variation	Degrees of freedom
Main plot:	
Between groups	2
Control v FAP gene carriers	1
No polyps v polyps	1
Between subjects within groups (error 1)	28
Main plot total	30
Sub-plot:	
Between substrates	3
Sugars v polysaccharides	1
Starch v arabinogalactan	1
Glucose v raffinose	1
Group × substrate interaction	6*
Error 2	80
Sub-plot total	89
Grand total	119

*This interaction was also split into its six single degree of freedom contrasts defined by the primary contrasts.

(blank) or 250 mg of starch (soluble potato starch, Sigma S2630), arabinogalactan (larch wood, Sigma A9029) glucose (Sigma, G8270) or raffinose (Sigma R250) were placed in 25 ml McCartney bottles. Incubation bottles were gassed with CO₂, sealed with a Suba seal and vented with a hypodermic needle. Duplicate incubations were performed in an orbital shaker (Gallenkamp) at 37°C and 125 rpm. Samples for SCFA analysis were removed after 24 hours.

SCFA ANALYSIS

The SCFAs were measured as previously described.¹⁴ Duplicate samples were de-proteinised with 20% metaphosphoric acid (2:1 w/v) containing 3 methyl valeric acid (50 mM) as an internal standard. Analysis was performed in duplicate by gas chromatography (Pye Unicam P4550, 1.5 m × 2 mm internal diameter column, Chromosorb 80/100 packing (Supelco Bellforte PA 16823 USA), N₂ carrier gas, flow rate 55 ml/min at 125°C).

STATISTICS

All statistics were performed by analysis of

variance with the Genstat V package for the mainframe computer. Differences between groups were tested by orthogonal contrasts (Table II). For the main part of the study the hypotheses were that: (a) there is no difference between controls and subjects with the FAP gene; (b) there is no difference between FAP gene carriers with and without polyps.

The other hypotheses tested were that: (c) there is no difference between controls and FAP gene carriers with no polyps; (d) there is no difference between controls and FAP gene carriers with polyps.

Also, where different substrates (glucose, raffinose, starch, and arabinogalactan) were used for in vitro incubations and tested within each subject, substrate effects were treated as a sub-plot. Hypotheses concerning differences between substrates and possible group × substrate interactions were tested by orthogonal contrasts (Table II). The contrast arabinogalactan × other carbohydrates was also tested.

Results

THE EFFECT OF ANAEROBIC STORAGE FOR SIX HOURS ON FAECAL SCFAS

Four separate stool samples from the same donor were stored at 4°C for six hours. Storage had no effect on the absolute amounts of molar proportions of SCFAs in faecal samples over a six hour period (Tables III and IV).

THE EFFECT OF ANAEROBIC STORAGE FOR 24 HOURS ON IN VITRO PRODUCTION OF SCFAS

The net production of SCFAs attributed to the fermentation of the added carbohydrate was calculated by subtraction of the corresponding blank value from the one with added carbohydrate. All the SCFA values presented are net values.

Storage for 24 hours before initiating incubation with the test carbohydrates had no significant effect on the SCFAs produced (Tables V and VI). There were, however, noticeable trends towards decreased butyrate and increased acetate after storage. To minimise differences due to storage in the main experiment, all samples were stored for similar periods of time (18-24 hours) before incubation.

FAECAL PH, PERCENTAGE DRY MATTER, SCFA CONCENTRATION, AND MOLAR RATIOS

There was no difference in the dry matter content of stool samples between the three groups (Table VII). Likewise there were no differences in the absolute concentrations or relative proportions of acetate, propionate, or butyrate (Table VIII). There were no differences in faecal pH.

TABLE III The influence of storage on faecal short chain fatty acids (SCFAs)

Storage time (h)	SCFA (mMol/kg faeces)				Molar ratio (mMol/mol)		
	Acetate	Propionate	Butyrate	Total	Acetate	Propionate	Butyrate
0	75.3	20.3	31.5	133.7	565	152	236
3	77.5	20.9	35.0	139.5	557	150	249
6	74.6	20.5	35.7	136.7	548	150	258
Pooled error	11.91	3.93	7.15	10.40	16.2	3.4	12.6

Total SCFA includes values for isobutyrate, isovalerate, and valerate (not presented).

TABLE IV Analysis of variance for data in Table III

Source of variation	Degrees of freedom
Stool sample	3
Storage time	2
Stool × storage time interaction (error)	6

TABLE V The effect of storage of stool samples for 24 h on the *in vitro* fermentation of carbohydrate

Substrate	SCFA (mMol/l in incubation fluid)						Molar ratio (mMol/mol)		
	Storage period (h)	Incubation period (h)	Acetate	Propionate	Butyrate	Total	Acetate	Propionate	Butyrate
Starch	0	24	46.6	9.8	40.4	96.9	480	102	417
Arabinogalactan	0	24	31.1	6.3	6.5	45.5	684	138	142
Glucose	0	24	47.6	8.7	47.8	103.9	458	84	460
Starch	24	24	54.0	9.3	30.4	93.7	577	99	324
Arabinogalactan	24	24	29.2	6.8	6.1	44.1	662	154	137
Glucose	24	24	69.2	8.3	27.2	104.5	663	80	261
Pooled error			4.43	0.64	5.09	4.07	47.5	3.4	47.7
Probability			NS	NS	NS	NS	NS	NS	NS

Abbreviations and footnotes as for Table III.

TABLE VI Analysis of variance for data in Table V

Source of variation	Degrees of freedom
Main plot:	
Stool sample	3
Storage time	1
Stool × storage time interaction (error 1)	3
Main plot total	7
Sub-plot:	
Substrate	2
Substrate × storage time interaction	2
Error 2	12
Total	23

TABLE VII Dry matter content of stools as determined by freeze drying to constant weights (differences are NS)

Group	Mean dry matter content of stool (% wet weight)		
	Mean	Range	SE
Control	30.0	20.0–56.6	2.94
FAP with no polyps	26.2	14.5–32.7	1.82
FAP with polyps	28.2	17.2–35.5	2.18

TABLE VIII The absolute amounts and molar ratios of SCFAs and pH in faeces

Group	SCFA (mMol/kg faeces)				Molar ratio (mMol/mol)			pH
	Acetate	Propionate	Butyrate	Total	Acetate	Propionate	Butyrate	
Control	76.3	29.4	34.5	155.4	520	185	193	6.46
FAP with no polyps	88.5	33.1	33.2	171.3	524	197	189	6.43
FAP with polyps	86.1	33.7	38.3	175.7	500	195	200	6.37
Pooled error	17.97	8.60	11.42	40.01	28.5	14.1	24.9	0.15

Abbreviations and footnotes as for Table III.

TABLE IX Absolute amounts and molar ratios of SCFAs produced after *in vitro* incubation for 24 h with starch, arabinogalactan, glucose, or raffinose

Substrate	SCFA (mMol/l in incubation fluid)				Molar ratio (mMol/mol)		
	Acetate	Propionate	Butyrate	Total	Acetate	Propionate	Butyrate
Control:							
Starch	50.7	10.1	18.0	78.5	664	121	218
Arabinogalactan	34.0	9.9	5.3	50.0	690	189	101
Glucose	49.6	10.8	21.2	82.7	617	129	255
Raffinose	52.4	9.8	20.0	83.7	631	117	249
No polyps:							
Starch	40.7	7.8	12.2	60.9	690	116	190
Arabinogalactan	27.8	8.9	5.2	42.4	653	209	124
Glucose	35.8	8.0	22.3	66.8	549	115	327
Raffinose	38.2	8.5	19.9	67.1	570	126	298
Polyps:							
Starch	54.2	4.6	4.0	62.4	878	70	59
Arabinogalactan	31.4	6.5	3.0	41.7	737	164	79
Glucose	58.8	5.1	10.3	74.0	797	67	139
Raffinose	56.6	6.2	12.7	75.6	754	83	166
Pooled error	3.65	1.54	2.66	4.72	39.34	18.72	31.04
Main plot contrasts:							
Polyps v no polyps	0.001	NS	0.011	NS	0.001	0.054	0.001
Control v gene	NS	NS	0.023	0.017	NS	NS	NS
Control v polyps	NS	0.028	0.002	0.072	0.002	NS	0.004
Control v no polyps	0.01	NS	NS	0.016	NS	NS	NS
Sub-plot contrasts:							
Polyps v no polyps between arabinogalactan and other carbohydrates	0.001	NS	0.048	NS	0.012	NS	0.014
Control v polyps between arabinogalactan and other carbohydrates	NS	NS	0.008	NS	0.01	NS	0.022

Abbreviations and footnotes as for Table III.

Figure 1: Sample time course of SCFA production from starch for one control subject.

COMPARISON OF THE FERMENTATION BY THE SUBJECT GROUPS

Comparison of SCFA production by the different subject groups showed considerable differences (Table IX, Fig 3). Compared with normal controls, gene carriers without polyps produced less total SCFA ($p=0.016$) with a reduction in the absolute amount of acetate ($p=0.01$) but no

Figure 2: Comparison of (A) net concentration and (B) molar ratios of SCFAs produced from starch, arabinogalactan, glucose, and raffinose after 24 hours incubation (means (SE)). Total SCFA includes values for isobutyrate, isovalerate, and valerate (not presented). Values presented are means for all study groups shown in Table IX with significance values from ANOVA and orthogonal contrasts.

Figure 3: Comparison of (A) net concentrations and (B) molar ratios of SCFAs produced after 24 hours incubation by controls, gene carriers with no polyps, and gene carriers with polyps (means (SE)). Total SCFA includes values for isobutyrate, isovalerate, and valerate (not presented). Values shown are means of all four carbohydrates shown in Table IX with significance values from ANOVA and orthogonal contrasts.

difference in the molar ratio of acetate, propionate, or butyrate.

Comparison, however, of normal controls and gene carriers with polyps showed a different pattern of changes. There was a slight non-significant decrease in the total SCFA produced by those patients with polyps ($p=0.072$). The absolute concentrations of both propionate ($p=0.028$) and butyrate ($p=0.002$) were decreased. The molar proportion of acetate was increased ($p=0.002$) whereas that of butyrate was decreased ($p=0.004$) and there was evidence of a slight, non-significant, fall in propionate ($p=0.067$). Gene carriers with and without polyps showed opposite changes in fermentation patterns when compared with normal controls. Consequently comparison of the two FAP groups showed considerable differences. There were no

differences in the total SCFA produced, but gene carriers with polyps produced significantly larger molar ratios of acetate ($p < 0.001$) and smaller ratios of butyrate ($p < 0.001$) and propionate ($p = 0.054$) than did those with no polyps.

INTERACTIONS BETWEEN THE SUBJECT GROUP AND SUBSTRATE ON FERMENTATION END PRODUCTS

There were relatively few significant interactions between subject group and substrates. The apparent effect of the presence of polyps on the SCFA pattern was different with arabinogalactan compared with other substrates. The reduction of butyrate in the presence of polyps that was noted for the other carbohydrates was not detected with arabinogalactan (Table IX, Fig 4). Likewise the corresponding increase in acetate with polyps with other carbohydrates was not as obvious with arabinogalactan. There were no other significant group $\times$ substrate interactions.

CORRELATION BETWEEN AGE OF ONSET OF POLYPS AND FAECAL BUTYRATE

Within gene carriers with polyps there was no relation between the age of onset of macroscopic polyposis and the faecal butyrate (absolute amounts or molar ratios) or its in vitro production (absolute amounts or molar ratios).

Discussion

Most studies in humans of dietary influences on the development of colonic cancer have relied on population epidemiological surveys or case-control studies. Both these methods have limitations and neither can prove causality. In case-control studies attributing a change in a variable to the cause, rather than the result, of the tumour is particularly difficult.

Familial adenomatous polyposis is a unique human model in which to study the development of colonic cancer. Affected subjects have a defect on chromosome 5¹⁵ that initiates adenomatous change but, in common with sporadic cancers, the colonic mucosal cells are thought to undergo at least four other genetic changes before a cancer can develop.¹⁶ Environmental factors of importance in the development of sporadic colonic cancer are likely to act by influencing this sequence of changes. Subjects with FAP show a wide spread of ages when polyps first develop.¹⁷ This suggests that there may be similar environmental effects in FAP. The model is advantageous as gene carriers can be identified before polyps develop¹²; as a result, changes that pre-date, and therefore may promote, polyp growth may be investigated. Furthermore it provides an ideal study group for future dietary intervention studies.

Although the epidemiological evidence suggests a protective role for dietary complex carbohydrate in the aetiology of colorectal cancer, this has not been proved. To this end, a possible mode of action has been sought and recent studies have focused on the production of SCFAs by colonic fermentation of carbohydrates.⁹⁻¹¹ These are present in considerable concentrations

Figure 4: Subject group by substrate interactions in the production of (A) butyrate and (B) acetate. Figures presented are molar ratios (SE). SCFA figures for other carbohydrates are means for starch, glucose, and raffinose shown in Table IX and significance values are from ANOVA and orthogonal contrasts.

in the faeces and approximately 300 mmol a day are produced.¹⁸ They are important in salt and water absorption from the colon,¹⁹ and butyric acid, one of the SCFAs, is the most important respiratory fuel for the colonic mucosa.¹⁸

The reduction in pH associated with the production of SCFAs has been proposed as a protective factor⁵ as a higher faecal pH has been noted in high risk populations.²⁰ Some studies have shown high faecal pH in persons with colonic cancer,²¹ although others have failed to confirm these findings.²² We detected no differences in faecal pH in our three subject groups suggesting that neither the FAP gene nor the presence of polyps influences faecal pH.

Interest has recently focused on the role of butyric acid in the development of colonic cancer. This compound has antineoplastic properties in vitro⁶ and decreased molar proportions have been found in faecal and enema samples from patients with colonic cancers and polyps.^{9,10} Furthermore decreased butyrate has been noted in similar patient groups with an in vitro model of caecal metabolism.¹¹ The apparently decreased caecal starch delivery in polyp formers²³ gives

further support to the hypothesis, as it seems that starch fermentation results in relatively larger concentrations of butyric acid.^{24 25}

We found no changes in the in vivo faecal SCFAs in subjects with FAP; furthermore we could not show any association of polyps with the faecal SCFA pattern. We did, however, show different SCFA production with the in vitro model. This apparent discrepancy has been noted by other workers¹¹ and is probably a consequence of the poor reflection of total SCFA production afforded by the faecal SCFA profile. Alternatively, it may represent the variability of SCFA production in vivo between subjects and the variability within subjects over time due to dietary factors. Whereas it is possible that different proportions of dry matter in stool samples could lead to different sizes of bacterial inoculum for in vitro culture, there were no such differences between the three groups in the present study, so this is unlikely to have been a confounding factor.

Our data, from the in vitro model, confirm earlier findings that different carbohydrates are fermented to give different patterns of SCFAs.^{14 24 25} This may in the future allow dietary manipulation of the intracolonic environment. We showed influences of both FAP gene carriage and the presence of polyps on the pattern of SCFAs produced. The FAP gene carriers without polyps produced less acetate and less total SCFA than normal controls. This has not previously been reported and the explanation for it is unclear. Previous workers have noted abnormalities in colonic cholesterol metabolism in FAP,²⁶ and changes in both faecal²⁷ and duodenal²⁸ bile acid profiles have been recorded. Bile acids are known to alter faecal microbial growth²⁹ and the influence of bile acids on fermentation may explain the changes noted. Alternatively in the light of recent evidence showing in vivo interactions between colonic fermentation of starch and bile acid transformations³⁰ it is possible that the altered fermentation in FAP gene carriers without polyps is a primary change that may then influence bile acid transformations and may explain the results of other workers. It is not possible to conclude from our data whether the altered fermentation has any role in the progression of polyps but it is possible that the interaction of fermentation and bile acid metabolism may decrease secondary bile acids that are known promoters of colonic cancers.³¹

Polyps also seem to affect the fermentation patterns of carbohydrates. The changes in SCFAs associated with polyps in our study are similar to those noted by other workers in enema⁹ and faecal samples¹⁰ and with in vitro models.¹¹ It is possible that the changes noted by these workers were secondary to the presence of polyps rather than a contributory factor in their development. Alternatively the FAP gene carriers may be a heterogeneous group in which the early appearance of polyps is linked with reduced colonic butyrate. We showed no correlation between the age of onset of polyps and the faecal butyrate or the in vitro production of butyrate. This might be tested through a prospective study in which FAP gene carriers without polyps are divided into high and low butyrate groups and

followed up until polyps develop.

Clausen *et al* considered the problem of causality by studying patients with sporadic neoplasms after colonic resection or endoscopic polypectomy and showed a reduction in the velocity of production of butyrate in affected patients.¹¹ As the tumours had been removed it is not immediately possible to ascribe their results to an effect of polyps or cancers. All their subjects, however, had undergone bowel purgation, colectomy, or endoscopic polypectomy. The influence of these procedures on the microbial activity is not known and this difference in experimental protocol may explain the apparent disagreement with our results.

In conclusion we have identified different fermentation patterns in persons with FAP and an effect of polyps on the in vitro fermentation of carbohydrates. Furthermore we have shown that different carbohydrates produce different patterns of SCFA and that there may be interactions between host genotype and colonic fermentation. The role of colonic fermentation on the initiation and progression of colonic polyps merits further investigation, as this process is amenable to dietary manipulation.

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