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ORIGINAL CONTRIBUTION

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The carbohydrate crystalean and colonic microflora modulate expression of glutathione S-transferase subunits in colon of rats

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Summary
Background Glutathione S-transferases (GSTs)* are an important class of phase II, predominantly detoxifying, enzymes. The supergene family is composed of several isoenzymes, hetero- and homodimers, with tissue specific distribution and levels of expression. The hypothesis is that a higher expression of individual proteins within a specific tissue may be associated with a decreased burden of exposure to reactive carcinogens and ultimately with a decreased cancer risk in this tissue.

Aims of the study Since nutrition is expected to contribute to the gene expression, it was the aim of this study to investigate the impact of dietary factors, especially resistant starch, and of the gut microflora, which may be influenced by diet, on the GSTs in colon cells of rats.

Methods For this, a technique using high pressure liquid chromatography was established with which for the first time GST isoenzymes were analysed in colon cells and compared to the levels of the corresponding proteins in the liver of the same rat.

Results It was found that colon cells contain mainly GST π and low amounts of μ but not GST α. In contrast, the predominant form of GSTs in the liver was α, then μ and hardly any π. Altogether, liver cells had approximately tenfold more total GSTs than colon cells. The feeding of "Crystalean", a retrograded, high amylose starch which alters the fermentation profile and the composition of the microflora, led to higher levels of GST π in the colon. Furthermore, the comparison of GSTs in colon cells of germ-free rats revealed they were much lower than those observed in rats with conventional microflora.

Conclusions These findings clearly demonstrate that the gut bacteria, or their metabolic products, enhance GST expression. The studies support the hypothesis that nutrition – by affecting the gut flora – may induce this potentially protective and important class of phase II enzymes in important tumor target cells.

Key words Glutathione S-transferase – crystalean – colon – diet – microflora

Introduction

Epidemiological data as well as experimental findings indicate that the human diet contains a variety of protective, anticarcinogenic compounds (13, 25). In general, the protective mechanisms of these structurally diverse anticarcinogens are poorly understood. They may act on various sites and at various stages of carcinogenesis (16, 17). Individuals might be protected against cell damaging processes that could lead to various diseases – especially cancer – when their chemoprevention systems, including glutathione S-transferase enzyme system, are efficient (3, 11). The glutathione S-transferases (EC 2.5.1.18) represent a family of isoenzymes that predominantly detoxify different exogenous and endogenous substances (3, 11). As multifunctional phase II enzymes, GSTs may protect against cancer by inactivating carcinogens (11, 23). According to their physical, chemical, structural, immunological and catalytic properties, cytosolic GSTs can be divided into different classes with accompanying subunits (e.g., class α – subunits 1 and 2; class μ – subunits 3 and 4; class μ – subunit 7) (15). It is well documented that activities, patterns of expression, and induction of these enzymes are species-, tissue-, age- and sex-specific (11). Different subunits show different but overlapping substrate selectivities and the specific expression of the subunits in the organs may also elucidate their role in detoxification of substrates (29). Although a large database on the associations of GSTs and cancer risk is available, indepth knowledge on mechanisms regarding colon cancer and nutritional impact is scarce, demanding further investigation. The present study had three main objectives, addressed in independent experiments: The aim of the first experiment was to explore the relationships between GST subunit levels and patterns in rat colon and liver of the same animal. Colon was chosen since it is an important target organ for diet-related tumors and liver for its detoxification activity. Comparison of different organs might give insights into the status of protection both of the organ and of the individual. In the second experiment, germ-free and conventional rats were used to assess the influence of the conventional rat microflora on induction of GSTs in colonic epithelial cells. There is extensive evidence to suggest that the gut microflora plays an important role in colon carcinogenesis by syntony of tumour indicators, promotors and anticarcinogens (8, 19, 27). The final experiment aimed to evaluate the modulatory role of diet on GSTs induction. Induction of GSTs closely correlates with tumor inhibitory activity in animal models; thus, GSTs play a central role in chemoprevention of carcinogenesis. They have been used as markers for screening potential chemopreventive agents (24, 28). As a result, GSTs induction is generally considered to be an event involved in cancer chemoprevention by diet (11, 28). Several dietary ingredients are known to be inducers of GSTs in rats and humans (11). Diets with high vegetable and fruit content as well as low in fat or high in fibre and calcium are associated with reduced cancer risk (4, 12). As one special factor related to risk reduction, the influence of complex carbohydrates was also investigated. Poorly digestible complex carbohydrates are metabolized by the gut microflora and yield different patterns of short chain fatty acids (n-butyrate, propionate and acetate). In particular, fermentation of starch in the colon is associated with elevate n-butyrate production (9). This is postulated to be an important protective agent in colon carcinogenesis via a number of possible mechanisms (1, 2, 8, 30). In the present study, rats were fed Crystalean, a retrograded amylomaize starch that is known to be poorly digested and is metabolised to high amounts of n-butyrate (21). In this experiment the animal model used comprised germ-free rats associated with human microflora to provide increased relevance to man (18, 19, 27). In addition, rats with conventional microflora were examined in comparison to germ-free animals to investigate the basic question of whether the colon microflora, probably by the action of its metabolites, may affect enzyme induction in the epithelial colon cells.

Materials and methods

Chemicals

Milli-Q water (Millipore, Eschborn, Germany) was used for preparing buffers or solutions. For the HPLC, HPLC grade acetonitrile (Merck, Darmstadt, Germany) and HPLC grade water (Baker, Griesheim, Germany) were used. Protein dye reagent concentrate was obtained from BIO-RAD Laboratories GmbH (München, Germany). All other chemicals were of the highest quality commercially available and from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany) unless otherwise stated.

Materials

An ultra-turrax T 25 (Janke & Kunkel GmbH & Co. KG, IKA-Labortechnik, Staufen i.Br., Germany) was used to homogenize cells. Amicon-Centricon tubes (Millipore, Eschborn, Germany) were used for cytosol concentration. For the HPLC a Vydac 218 TP C18 column (250 x 4.6 mm, 5 µm dp, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany), two HPLC pumps (no. 510 and 501), an injector (U6K) and UV detector (486) were used (Waters, Eschborn, Germany); the integration was performed with Chromatography Software (Version 2.10, Millenium).

Animals and Diets

Experiment I

For the experiments comparing GSTs in liver and colon, three groups of male Sprague Dawley (SD) rats (151-

325 g body weight, 36-46 days old) were used. The first and the third group were fed the standard diets Altromin Haltung and subsequently C 1000 (Altromin GmbH & Co. KG, Lage, Germany) for 7 and 11 d, respectively; all animals were obtained from breeding stock at the Federal Research Centre. The second group of rats, bought from Charles River Wiga (Sulzfeld, Germany), were fed the diet C 1000 for 9 d (for diets see Table 1).

Experiment II:

For assessing the impact of the gut flora on GST protein levels, conventional (CV) microflora rats and germ-free (GF) male and female F344 rats were donors of colon cells (4 male CV rats: 215-277 g, 2 female CV rats: 176-190; 4 male GF rats: 249-300 g, 2 female GF rats: 187-189; 89 days old). Rats were given HR diet for 42 d.

Experiment III

To study the influence of diet on GSTs, colon cells from three different feeding groups of female human flora associated (HFA)-F344 rats were examined: the first group received 46 % (w/w) maize starch (MS), Dottori Piccioni Milan, Italy, the second group 36 % (w/w) MS and 10 % retrograded amylomaize starch Crystalean (CL), Williamsons, Ltd., Ireland, the third group 31 % (w/w) MS and 15 % CL to the standardised diets for 29 d (MS group: 257-265 g, 154-168 days old; 10 % CL group: 235-258 g, 175 days old; 15 % CL group: 240-276 g, 154-168 days old).

Origin and maintenance of HFA and germ-free animals has been described previously (10). All rodents were kept on a 12 h artificial light (06.00-18.00)/12 h dark cycle, at room temperature (19-23 °C) and relative humidity in the range of 45-65 %, and received water and diets ad libitum (for diets see Table 2).

Isolation of cells, preparing of cytosol and assays

Rat liver hepatocytes were isolated by in situ collagenase perfusion as described previously (22). Rat colon cells were isolated by protease digestion (20). Immediately after the isolation, cells were counted in a haemocytometer and vitality was determined with trypan blue. The following steps were performed at 4 °C: defined aliquots were homogenised with the ultra-turrax at the highest speed for one minute in a buffer containing 0.020 M Tris-HCl, 0.250 M saccharose, 0.001 M DTT, 0.001 M PMSF and 0.001 M EDTA (pH 7,4). Subsequently the cytosols were prepared by ultracentrifugation (105,000 g, 75 min). Protein was determined in the cytosol by the method of Bradford (7) with protein dye reagent concentrate from BIO-RAD, using bovine serum albumin as standard. GST subunits were further processed as described previously (6) with slight modifications. In brief, the enzymes were isolated and purified by affinity chromatography. For this the cytosolic samples (aliquots containing the cytosol prepared from about 15 x 10^6 colon cells or 3 x 10^6 liver cells, respectively) were dispersed onto columns filled with S-hexylglutathione sepharose 6B, washed and then eluated with 6 ml of a buffer containing S-hexylglutathione. The eluates were concentrated by membrane centrifugation to about 300 µl and then subjected to high performance liquid chromatography (50 µl injection volume) with a CH_3CN (TFA)/H_2O (TFA) gradient program on RP 18. Detection was by UV absorption at 214 nm. Quantitative calibration was performed with standard proteins, kindly donated by J.J.P. Bogaards, Zeist, the Netherlands.

Table 1 Formulation of Altromin Haltung (AH) and C 1000 diets (g per kg diet)

Ingredient	AH	C 1000
Fat	40	51
Protein	190	173
Minerals	70	55
Fibre	60	40
DL-methionine	3	11
Lysine	9	17
Calcium	9	10
Phosphorus	7	8
Sodium	2	2
Magnesium	2	1
Vitamin A	15,000 I.E.	15,000 I.E.
Vitamin D_3	600 I.E.	500 I.E.
Vitamin E	0.075	0.164
Copper	0.005	0.006

Table 2 Formulation of maize starch (MS), Crystalean (CL) and high risk (HR) diets (g per kg diet)

Ingredient	MS	CL 1	CL 2	HR
Maize starch	460	360	310	231
Crystalean starch	0	100	150	0
Sucrose	0	0	0	231
Sodium caseinate	231	231	231	254
Lard	0	0	0	204
Corn oil	231	231	231	26
AIN vitamin mix	12	12	12	12
AIN minerals (low Ca^{2+})	40	40	40	40
Choline bitartrate	2	2	2	2
Calcium carbonate	0	0	0	0
Cellulose	21	21	21	10
DL-methionine	3	3	3	0

Results

Nine animals were simultaneously worked up for isolation of intact cells from the liver and from the epithelium layer of the colon. A typical HPLC chromatogram of GST protein subunits of these animals is shown in Fig. 1. According to the commercially available standards, subunits 1, 2, 3, 4 and 7 could be identified. Some peaks of liver cells could not be paired to standards. There were no significant differences between the three groups of rats with respect to amounts of quantifiable GST subunits (two sided unpaired t-test); thus, results for the animals were pooled. It is apparent that the liver contained more GSTs than the colon. On a quantitative basis these differences were approximately threefold higher for GSTs protein/mg cytosolic protein and eighteenfold higher for GSTs protein/10^6 cells. The figure also shows that different subunits were expressed in the two tissues: as is apparent from the sample chromatogram of Fig. 1 and more in detail for different rats in Fig. 2 and 3, colon cells contained mainly GST π (0.28 µg ± 0.05 µg/10^6 cells, 3.35 µg ± 0.34 µg/mg cytosolic protein; means ± SEM, n = 9) followed by GST m (subunit 3: 0.10 µg ± 0.02 µg/10^6 cells, 1.20 µg ± 0.19 µg/mg cytosolic protein; means ± SEM, n = 5; and subunit 4: 0.13 µg ± 0.02 µg/10^6 cells, 1.57 µg ± 0.16 µg/mg cytosolic protein; means ± SEM, n = 9) and hardly any GST a (subunit 2: 0.14 µg ± 0.04 µg/10^6 cells, 1.29 µg ± 0.11 µg/mg cytosolic protein; means ± SEM, n = 3; and subunit 1: not detectable) were found. In contrast the liver contained mainly subunits 1 (3.01 µg ± 0.53 µg/10^6 cells, 6.08 µg ± 0.91 µg/mg cytosolic protein; means ± SEM, n = 8) and subunit 2 (2.52 µg ± 0.53 µg/10^6 cells, 4.73 µg ± 0.20 µg/mg cytosolic protein; means ± SEM, n = 8) followed by subunit 4 (1.81 µg ± 0.45 µg/10^6 cells, 3.25 µg ± 0.36 µg/mg cytosolic protein; means ± SEM, n = 8) and subunit 3 (1.66 µg ± 0.44 µg/10^6 cells, 3.15 µg ± 0.69 µg/mg cytosolic protein; means ± SEM, n = 8) with hardly any subunit 7.

A second study was to determine influences of the gut microflora on GST levels by comparing rats with conventional microflora and germ-free bred rats: there were significant higher levels of the π form (subunit 7), but not the µ form, in colon cells from conventional F344 rats compared with cells from germ-free animals (GST subunits (µg/mg cytosolic protein): GF group: 1.00 ± 0.10 (subunit 3), 1.23 ± 0.08 (subunit 4), 4.90 ± 0.23 (subunit 7); CV group: 1.10 ± 0.19 (subunit 3), 1.36 ± 0.19 (subunit 4), 7.38 ± 1.07 (subunit 7); means ± SEM, n = 6; p 0.05, one sided unpaired t-test; see Fig. 4). Sex specific differences were not detectable.

Finally, a third study was aimed at investigating the effect of a resistant starch (retrograded amylomaize starch Crystalean) on its effectiveness in modulating the GST enzymes in the colon. The Crystalean fed HFA F344 rats were compared to a group of rats fed digestible maize starch. The Crystalean feeding caused significant, three-

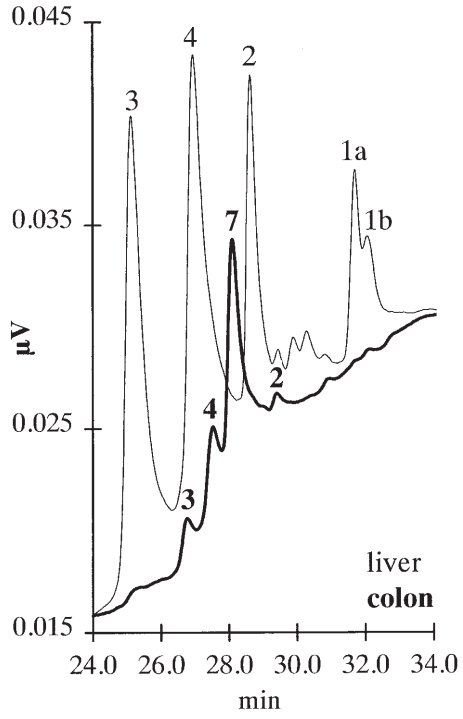

Fig. 1 HPLC chromatograms of affinity-purified GST isoenzymes of male SD rat colon and liver. The HPLC conditions are described in the materials and methods. X axis, retention time (min); Y axis, absorbance at 214 nm. The identity of each peak is indicated as number of GST subunit.

fold higher levels of GST π in comparison to the digestible starch fed group (GST π (µg/10^6 cells): MS group: 0.12 ± 0.02; 10 % CL group: 0.39 ± 0.13; 15 % CL group: 0.49 ± 0.13; (means ± SEM, n = 3); p <0.05, different from maize starch group, one sided unpaired t-test; see Fig. 5. GST π (µg/mg cytosolic protein): MS group: 4.65 ± 0.26; 10 % CL group: 5.93 ± 1.00; 15 % CL group: 11.76 ± 0.50; (means α SEM, n = 3); p <0.001, different from maize starch group, one sided unpaired t-test; see Fig. 6).

Discussion

This is the first time – to our knowledge – that GST subunits have been (1) separated and quantified by HPLC in rat colon cells, (2) compared to the levels in the liver and (3) additionally quantified on the basis of cell number. The GST values expressed as µg/10^6 cells are considered to be more accurate than µg/cytosolic protein, since it is expected that environmental factors can enhance general protein content in parallel to GSTs induction. Nevertheless, we also quantified GSTs on the basis of protein content to enable a comparison with literature data. To determine the cytosolic protein we chose the method of Bradford (7) since this is subject to less inter-

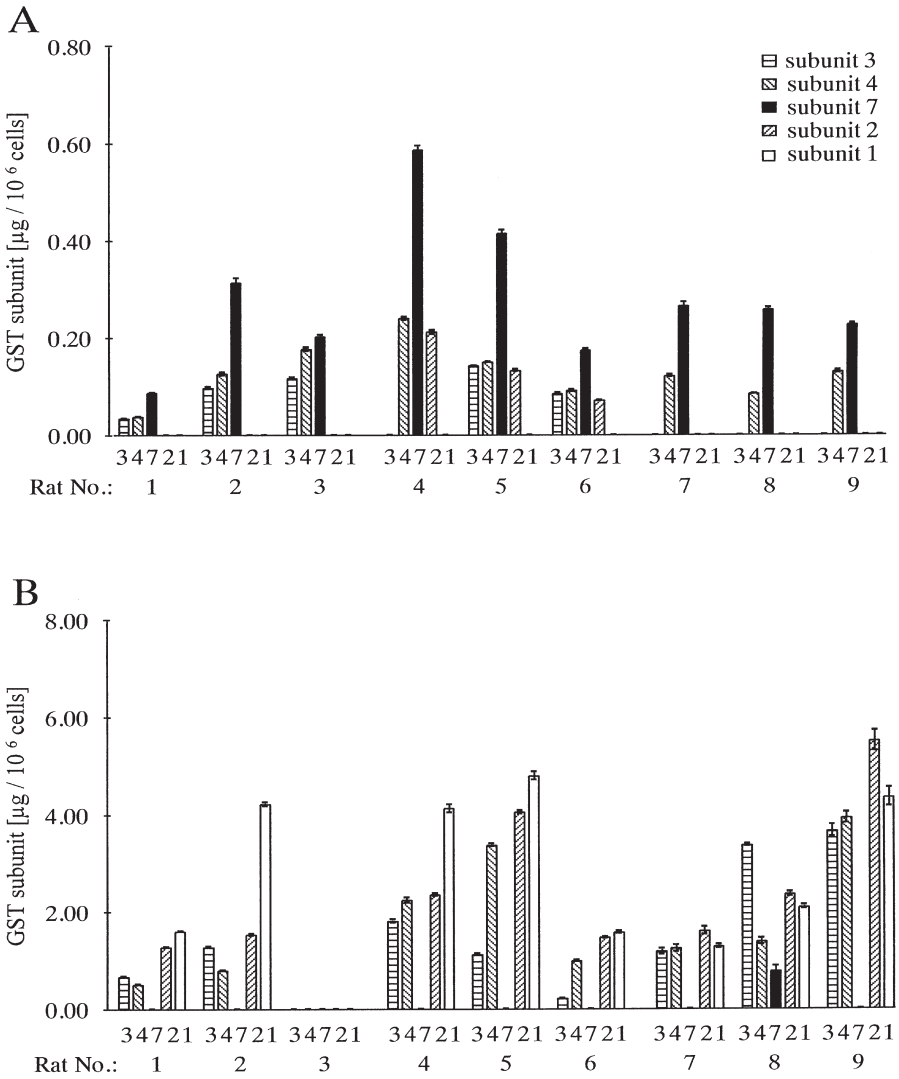

Fig. 2 Diagrams of male SD rat colon (A) and liver (B) GST subunits. Values are means from three determinations; basis cell number.

Table 3 GST levels in liver and colon cells of rats

GST		GST [µg/mg protein]				
subunit	class	liver			colon	
1	α	6.08±0.91[1]	4.58±0.10[2]	11.580±1.610[3]	n.n[1]	0.070±0.030[3]
2		4.73±0.20[1]	4.57±0.14[2]		1.29±0.11[1]	
3	µ	3.15±0.69[1]	5.31±0.17[2]	47.530±2.530[3]	1.36[1]	3.860±0.440[3]
4		3.25±0.36[1]	7.17±0.18[2]		1.52±0.44[1]	
7	π	1.65±0.23[1]	n.d.[2]	0.090±0.058[3]	3.61±0.45[1]	1.397±0.217[3]

[1] This study; means ± SEM, n = 8 (liver), n = 3 (colon) (except for GST π in the liver, in this case n = 1); SD-rats; protein determination according to Bradford; quantification of GST by HPLC;
[2] Bogaards et al., 1990; means ± SEM, n ≥ 5; F344 rats; protein determination according to Lowry; quantification of GST by HPLC;
[3] Nijhoff et al., 1992; means ± SEM, n = 8; Wistar rats; protein determination according to Lowry; quantification of GST by immunoblotting

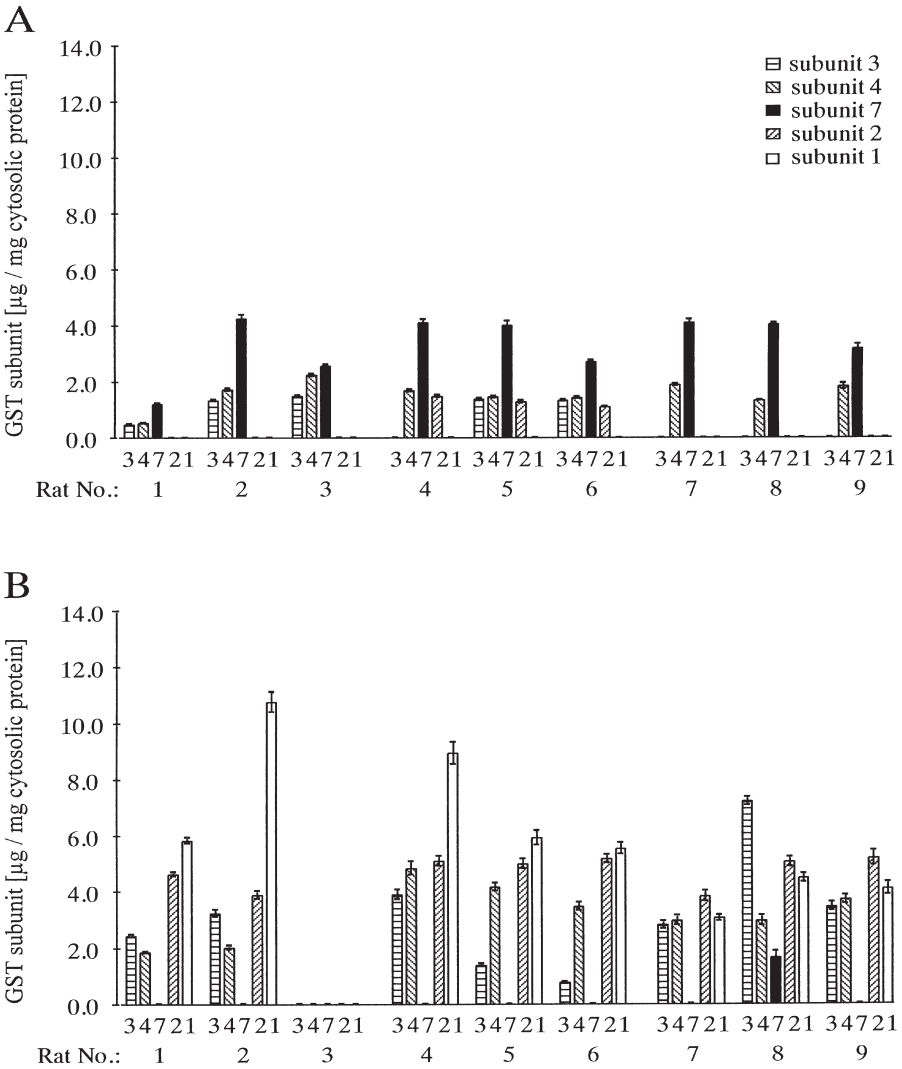

Fig. 3 Diagrams of male SD rat colon (A) and liver (B) GST subunits. Values are means from three determinations; basis protein content.

ference with, e.g. potassium and magnesium ions, EDTA, Tris, thiol reagents, carbohydrates and for being less time consuming (7) than the method of Lowry (14).

Our data regarding GST subunits in rat liver are consistent with literature data (5) (see Table 3). There are no literature data for a HPLC determination of GST subunits in colon cells of rats. Immunochemical detection of GSTs in rat colon and liver by Nijhoff et al. (18) revealed that our results are of the same order of magnitude – bearing in mind the differences in GSTs and protein determinations and stock of animal. Unfortunately, there is no differentiation between the GST subunits within the GST classes. Thus alterations of GSTs within a class may be disguised, if being of same amounts in opposite directions, for example. This is one of the advantages of the HPLC method, recognizing changes of all GST subunits in one chromatogram.

When comparing GSTs in liver and colon cells in individual rats we found that animals with high total GST

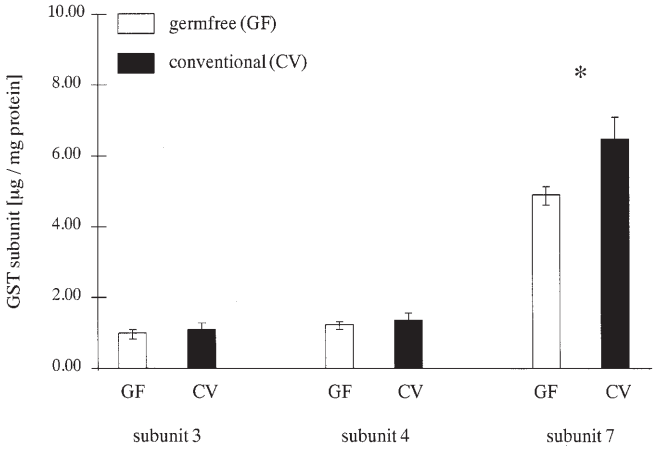

Fig. 4 Comparison of GST subunits in colon cells of F344 rats with germ-free or conventional colon microflora: rats with conventional microflora have significant higher levels of the GST subunit 7 as opposed to germ-free animals ± means (SEM, n = 6, * p <0.05, one sided unpaired t-test).

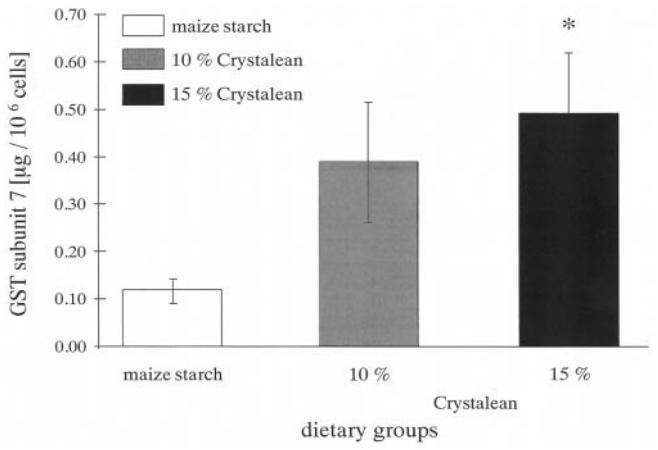

Fig. 5 Effect of complex carbohydrates in the diets of female HFA-F344 rats on levels of GST subunit 7 in colon cells (means± SEM, n = 3, * p < 0.05, *** p <0.001, different from maize starch group, one sided unpaired t-test); basis cell number.

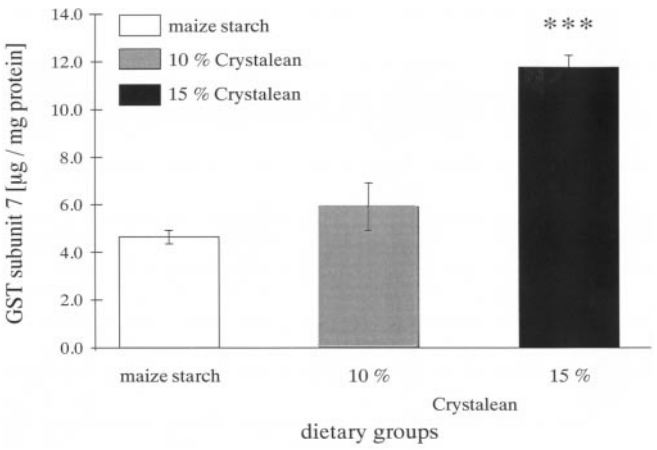

Fig. 6 Effect of complex carbohydrates in the diets of female HFA-F344 rats on levels of GST subunit 7 in colon cells (means ± SEM, n = 3, * p <0.05, *** p <0.001, different from maize starch group, one sided unpaired t-test); basis protein content.

levels in colon tend to show also high levels in liver and vice versa for the majority of rats (n = 7). The level of GST π appeared to be dependent on the gut microflora: significant higher levels of the π form were detected in colon cells of rats with a conventional microflora as opposed to germ-free animals. Furthermore, dietary factors can increase this induction. Rats fed the amylase resistant starch crystalean – a retrograded amylomaize starch – showed significant higher levels of GST π in comparison to animals fed digestible maize starch. The GSTs induction was associated with less DNA damage (30). One explanation for the mechanism may be that complex carbohydrates are fermented by the colon microflora to yield increased levels of short chain fatty acids as n-butyrate, propionate, and acetate and moreover in this mixture to higher yields of n-butyrate (Rowland, consolidated progress report AIR 2-CT94-0933). Furthermore, n-butyrate may be an important protective agent in colon carcinogenesis (2, 8, 30). It has been shown to induce GST π in the human tumor cell line Caco-2 (28).

In conclusion, our study shows that, through modulating the gut microflora, glutathione S-transferase levels could be modulated in colon cells. Nutritional habits, such as consuming special complex carbohydrates, may prevent colon from the genotoxic impact of carcinogenic factors through the mechanism of GSTs induction.

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***Abbreviations** GSTs, glutathione S-transferases; SD, Sprague Dawley; HR, high risk; LR, low risk; CV, conventional; GF, germ-free; HFA, human flora associated; MS, maize starch; CL, Crystalean.

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