Alterations of the hepatic xenobiotic-metabolizing enzymes by a glucosinolate-rich diet in germ-free rats: influence of a pre-induction with phenobarbital

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Germ-free growing rats were fed on a glucosinolate-rich diet (rapeseed-meal-based) and compared with counterparts fed on a glucosinolate-free diet (soya-bean-meal-based), both diets being isonitrogenous and isoenergetic. For each diet half the animals received phenobarbital in drinking water as an inducer of xenobiotic-metabolizing enzymes. Some of the usual deleterious glucosinolate-linked effects, i.e. kidney hypertrophy and reduction in growth and feed intake, were followed and three of the major hepatic xenobiotic-metabolizing enzymes were investigated. Growth rate, dietary intake and kidney weight were not altered by glucosinolates in the absence of intestinal microflora, whether the animals were treated with phenobarbital or not. As far as the hepatic xenobiotic-metabolizing enzymes are concerned, the specific level of cytochrome P450 and the specific activities of glutathione-S-transferase (*EC* 2.5.1.18) and UDPglucuronosyltransferase (*EC* 2.4.1.17) remained unchanged in rats receiving the glucosinolate-rich diet compared with the control animals. Despite the low dose given, phenobarbital displayed its usual inducing effect on all three enzymes, similar whatever the diet. A previous counterpart experiment performed with conventional animals had shown that glucosinolate feeding led to large alterations of the variables herein studied, some of these modifications being hugely enhanced by a phenobarbital treatment. Therefore, the present results obtained on germ-free animals prove that alterations of the xenobiotic-metabolizing enzymes induced by glucosinolates are somehow mediated by the intestinal microflora. Furthermore, the involvement of those enzymes in glucosinolate toxicity definitely requires the presence of the intestinal microflora.

Glucosinolates: Germ-free rats: Xenobiotic-metabolizing enzymes

Cruciferous vegetables (e.g. rape, turnip, cabbage) contain glucosinolates (GLS) that are not toxic *per se* but when broken down by intestinal microflora (Nugon-Baudon *et al.* 1988) lead to well-known toxic effects. Growth depression, dramatically reduced feed intake, enlargement of target organs (liver, kidneys, thyroid) and depletion of thyroid hormones plasma levels are the main side-effects observed among different animal species (Bourdon *et al.* 1981; Martland *et al.* 1984; Vermorel *et al.* 1987). In man, epidemiological surveys show a correlation between endemic goitre and consumption of cruciferous vegetables (Michajlovskij *et al.* 1969; Mitjavila, 1986) whereas experimental studies are more confused; Greer & Astwood (1948), Greer & Deeney (1959) and Langer *et al.* (1971) have demonstrated the goitrogenic properties of cruciferous vegetables and purified glucosinolates or glucosinolate derivatives but the findings of McMillan *et al.* (1986) are not in total agreement with these statements.

Epidemiological data (Anon. 1982) and experimental studies (Stoewsand *et al.* 1978, 1988) seem to indicate that cruciferous vegetable consumption is linked with a reduction

in the incidence of cancer at several sites in humans as well as in chemically-induced tumours in laboratory rodents.

Consequently, several authors have investigated the possible influence of cruciferous vegetables on the intestinal or hepatic detoxication system (xenobiotic-metabolizing enzymes; XME; Wattenberg, 1971; McDanell *et al.* 1989; Nugon-Baudon *et al.* 1990). Although results may vary depending on the strain of rat and the experimental design (Miller & Stoewsand, 1983), it seems that GLS-rich diets deeply modify both phase I (cytochrome P450) and phase II (transferases) enzymes.

It was then shown that the pre-induction of phases I and II by phenobarbital (PB) enhances the toxicity of a GLS-rich feed in rats, with the exception of thyroid disorders and liver hypertrophy, which are not different from those of non-induced animals (Nugon-Baudon *et al.* 1990).

In view of these findings, the aim of our work was to investigate whether the intestinal microflora is involved in the XME modifications induced by GLS and to determine whether the enhancement of GLS toxicity by PB treatment requires the presence of the intestinal microflora.

We, therefore, studied the influence of a GLS-rich diet (rapeseed-meal-based) *v.* a diet devoid of GLS (soya-bean-meal-based) in germ-free rats with or without PB treatment. Several microsomal hepatic XME were tested: total cytochrome P450 concentration, glutathione-S-transferase (*EC* 2.5.1.18) and UDPglucuronosyltransferase (*EC* 2.4.1.17) specific activities. As far as the toxic effects of GLS are concerned (anatomical and hormonal disorders), we shall only report here the findings relating to the variables which showed changes which were enhanced by PB in conventional animals, i.e. growth, dietary intake and kidney weight. Other observations (liver and thyroid weight, thyroid hormones alterations) were very similar to those already found and described elsewhere (Nugon-Baudon *et al.* 1988).

EXPERIMENTAL

Chemicals

Chemicals were obtained from the following sources: trizma-hydrochloride (Tris-HCl), DL-dithiothreitol (DTT), phenylmethylsulphonylfluoride, ovalbumine (chicken egg, grade V), Folin and Ciocalteu's phenol reagent, sodium dithionite, glutathione (reduced form), 1-chloro-2,4-dinitrobenzene (grade I), $MgCl_2$, chloramphenicol (CAP) and sodium salt of uridine 5′-diphosphoglucuronic acid (UDPGA) were from Sigma (La Verpillière, France). Potassium–sodium tartrate, $Cu_2(SO_4)_3$ and Na_2EDTA were obtained from Merck (Nogent-sur-Marne, France). ^{14}C-labelled CAP, labelled in the acetyl moiety with a specific activity of 54 mCi/mmol, was obtained from Amersham (Les Ulis, France). Scintillation fluid (Insta-Gel) was obtained from Packard (Rungis, France). CO and isoamyl acetate were obtained from Prolabo (Paris, France). PB as a 20 g/l drinkable solution was obtained from Avicopharma (Luzarches, France). All other chemicals used were of analytical grade.

Experimental diets

Two semi-synthetic diets were used, being both isonitrogenous and isoenergetic (Table 1). The protein fraction was supplied by soya-bean meal in the GLS-free diet (SM diet), whereas the GLS-rich diet contained 390 g dehulled 00 DARMOR rapeseed meal/kg (RM diet).

The GLS distribution in the rapeseed meal was analysed following an HPLC method (Anon. 1990; Table 2). The amount of total intact GLS in the meal was 42·75 μmol/g dry matter.

Table 1. *Composition of diets (g/kg)*

	Rapeseed-meal diet	Soya-bean-meal diet
Rapeseed meal (Darmor 00)	390·00	
Soya-bean meal 50		276·00
Maize starch	531·40	576·00
Maize oil	20·00	20·00
Lysine hydrochloride	0·60	
Vitamin mixture	18·00	18·00
Mineral mixture	40·00	40·00
Cellulose		70·00
Protein (N × 6·25; g/kg dry matter)	145·00	150·00
ME (kJ/kg dry matter)	15270	15440

Table 2. *Glucosinolate (GLS) content of irradiated Darmor rapeseed meal (μmol/g dry matter)*

Dry matter content (g/kg)	921
Total alkenyls	38·80
Progoitrin	25·11
Gluconapoleiferin	1·36
Gluconapin	8·29
Glucobrassicanapin	3·13
epi-Progoitrin	0·52
Glucoalyssin	0·40
Total indolyls and aromatics	3·95
Sinalbin	0·17
Gluconasturtin	0·23
4-hydroxy-glucobrassicin	2·86
Glucobrassicin	0·40
Neo-glucobrassicin	0·13
4-methoxy-glucobrassicin	0·17
Total GLS	42·75

Pelleted diets were packed in double-vacuum bags and sterilized by gamma-irradiation at 40 kGy.

Animals

Germ-free male Fischer 344 rats weighing 100–120 g at the beginning of trials and reared in Trexler-type isolators fitted with a rapid transfer system (La Calhène, Vélizy-Villacoublay, France) were used. All animals were produced by our rearing unit.

Rats were randomly distributed in two isolators (four cages per isolator and four animals per cage). In each isolator, one group of eight rats was fed on the SM diet and the other group on the RM diet. One isolator housed the PB-induced animals and the other housed the non-induced animals.

Room temperature was 21° and photoperiods of 12 h were used. Animals were fed *ad lib.* and given sterilized (120°, 20 min) tap water to drink. Feed intake and weight gain were measured twice weekly.

Induction procedure

PB was given in drinking water. The dose prescribed in the literature usually ranges from 0·7 to 1·0 g/l (Singh & Wiebel, 1979; McDanell & McLean, 1984; Nugon-Baudon *et al.* 1990). A preliminary set of experiments using that dose had led to the death of all germ-free animals, therefore, we lowered it to 0·1 g/l.

The induction procedure was started 3 d before the distribution of the experimental diets and was continued thereafter.

Sample collection

After 21 d, rats were knocked unconscious and immediately killed by cervical dislocation. Kidneys were removed and weighed after removal of the surrounding fat. Hepatic microsomes were prepared as described by Ryan *et al.* (1978).

Microsomal enzyme assays

Microsomal protein concentrations were determined according to the method of Lowry *et al.* (1951), using ovalbumin as a standard.

Cytochrome P450 was assayed by measuring the different spectra of microsomal preparations between 400 and 500 nm. Microsomal preparations were diluted to 0·5 and 0·8 mg protein/ml respectively for induced and non-induced rats, using a buffer containing 0·05 M-Tris-HCl, 10 mM-EDTA, 1 mM-DTT, 0·25 M-sucrose, pH 7·4. The diluted preparations were placed in both the sample and the reference cells and CO was bubbled through the two cuvettes for about 30 s to obtain saturation. Reduction of samples was effected with a few milligrams of solid sodium dithionite (Omura & Sato, 1964).

Glutathione-S-transferase activity was assayed according to the spectrophotometric method of Habig *et al.* (1974) using 1-chloro-2,4-dinitrobenzene except that the microsomal fraction was used instead of the cytosolic fraction (Nugon-Baudon *et al.* 1990).

UDPglucuronosyltransferase activity was measured by following the radiometric method of Young & Lietman (1978) using ^{14}C-labelled CAP. In a final volume of 0·15 ml the reaction mixture contained: 25 mM-Tris-HCl (pH 8·0 at 37°), 4 mM-$MgCl_2$, 30 mM-UDPGA, 15 mM-CAP in ethanol (specific activity 0·1 mCi/mmol) and microsomal preparation containing 1·0–2·0 mg proteins. Incubation lasted for 2 h at 37°. The extraction procedure using isoamyl acetate was performed only twice since a third extraction did not improve the recovery. All counts were expressed as disintegrations/min after correction for quenching and control counts obtained with the boiled enzyme (Scintillation fluid counter KONTRON SL30).

The specific activities of both transferases were defined as the amount of enzyme catalysing the formation of 1 nmol product/min per mg protein under the conditions of the assay.

All assays were performed in duplicate.

Statistical analysis

Results are expressed as means with their standard errors (n 8). Each variable was studied using a two-way classification analysis (Snedecor & Cochran, 1967). Significance was accepted when $P < 0·01$.

RESULTS

PB had a significant depressing effect on the growth of germ-free animals whatever the diet (Fig. 1). Final weight gains after 21 d of the experiment were 102 (SE 6) and 82 (SE 4) g for SM and PB–SM rats respectively. As far as the RM diet is concerned, RM animals gained 122 (SE 12) g whereas their PB-induced counterparts gained 102 (SE 13) g. The difference observed between RM and SM diets, whether the rats received PB or not, was significant

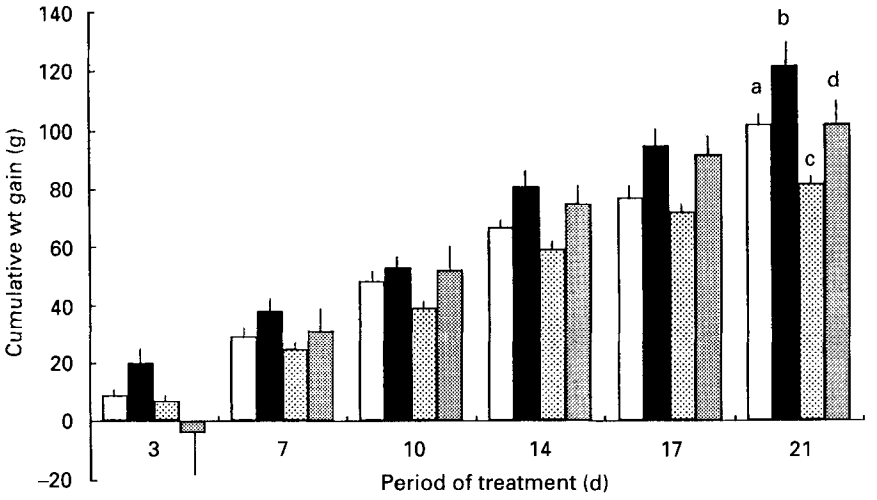

Fig. 1. The effect of diet and phenobarbital (PB) induction on the growth curve in germ-free rats. (□), Soya-bean-meal diet without PB; (■), rapeseed-meal diet without PB; (▨), soya-bean-meal diet with PB; (▩), rapeseed-meal diet with PB. Values are means with their standard errors represented by vertical bars. a, b, c, d Mean values for final weight gains with unlike superscript letters were significantly different ($P < 0.01$); groups which were different in both diet and PB induction were not compared. For details of diets and procedures, see Table 1 and pp. 349–350.

Table 3. *The effect of diet and phenobarbital (PB) induction on three hepatic xenobiotic-metabolizing enzymes in germ-free rats**

(Mean values with their standard errors for eight rats per group)

Diet...	Without PB				With PB			
	Soya-bean meal		Rapeseed-meal		Soya-bean meal		Rapeseed-meal	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cytochrome P450 (nmol/mg microsomal proteins)	0·85[a]	0·17	0·68[a]	0·15	2·44[b]	0·43	2·27[b]	0·33
Glutathione-S-transferase (*EC* 2.5.1.18; nmol/min per mg microsomal proteins)	30·0[a]	8·9	31·4[a]	6·6	46·9[b]	4·6	56·9[b]	12·5
UDPglucuronosyltransferase (*EC* 2.4.1.75; pmol/min per mg microsomal proteins)	149·6[a]	15·0	153·3[a]	54·4	1291·6[b]	380·8	1039·2[b]	137·9

[a,b] Within each line, means with unlike superscript letters were significantly different ($P < 0.01$); (groups which were different in both diet and PB treatment were not compared).

* For details of diets and procedures, see Table 1 and pp. 349–350.

and always in favour of the RM diet. PB had no influence on feed intake whatever the diet; values obtained from the consumption data for the whole group were respectively 18 *v.* 19 g/rat per d for SM and PB-SM animals and 14 *v.* 15 g/rat per d for RM and PB-RM animals. In contrast, these findings seem to indicate that RM diet had a slight reducing effect on feed intake, whether the animals were PB-induced or not. On the whole, RM diet improved the dietary efficiency compared with the control SM diet.

The calculated amount of GLS ingested daily was 217 and 232 μmol/rat for non-induced and PB-induced groups respectively.

Kidney weight, expressed as g/kg body weight, was similar for the SM and RM groups: 8·2 (SE 0·5) *v.* 8·4 (SE 0·3) respectively. PB treatment led to a significant hypertrophy, the intensity of which was similar irrespective of diet: 13·8 (SE 0·6 and 14·6 (SE 0·7) for PB–SM and PB–RM rats respectively.

PB increased the level of cytochrome P450 in the liver; the mean concentrations were 187 and 233% higher for PB–SM and PB–RM groups respectively compared with their respective non-induced counterparts (Table 3). Similarly, the specific activities of both transferases were significantly enhanced: +56 and +81% for glutathione-S-transferase and +763 and +578% for UDP glucuronosyltransferase in PB–SM and PB–RM groups respectively compared with the SM and RM groups (Table 3). The nature of the diet did not significantly affect either cytochrome P450 concentration or the specific activities of transferases. Furthermore, there was no significant interaction between the RM diet and the chemical inducer, i.e. the PB induction pattern was not modified by the presence of GLS in the diet and vice versa.

DISCUSSION

Greer & Deeney (1959) observed a decrease in rapeseed meal toxicity in conventional rats when treated orally with antibiotics. Our findings support this early foresight of the responsibility of intestinal microflora in GLS toxicity. They confirm findings obtained in a more recent experiment (Nugon-Baudon *et al.* 1988), i.e. the GLS-rich diet neither decreases growth nor alters kidney weight when animals are devoid of intestinal microflora. In the present experiments we even found a slight advantage of the RM diet compared with the SM diet as far as growth curve and dietary efficiency are concerned.

PB induction slightly decreased the growth rate and led to severe kidney hypertrophy. These deleterious effects, the nature and intensity of which were independent of the diet, were associated with the inclusion of 0·1 g PB/l in drinking water. In our preliminary experiments, 100% of the germ-free rats had died within 2 weeks after they had been given PB doses usually administered to conventional rats, i.e. 0·7–1·0 g/l (Singh & Wiebel, 1979; McDanell & McLean, 1984; Nugon-Baudon *et al.* 1990). Consequently, it seems that rats are extremely sensitive to PB oral treatment when they are devoid of intestinal microflora. Similar findings have been reported in the literature; in a long-term experiment designed to study the effect of PB in promoting liver tumourigenesis, Mizutani & Mitsuoka (1988) observed a lethal effect of dietary PB in young germ-free CH3/He mice but not in their conventional counterparts. However, to our knowledge, such phenomena have not been described in germ-free rats. Hietanen & Pelkonen (1979), while investigating several XME activities in germ-free rats, did not observe any toxicity of PB administered intraperitoneally for 5 d. Gustafsson & Persson (1975) even suggested that the germ-free status reduces pharmacological effects of barbiturate since they observed a reduced sleeping time in germ-free rats after a single intraperitoneal injection of pentobarbital. These divergent findings point out that barbiturate toxicity is a very complex issue. Further investigations are required to explain in which way the metabolic fate of these molecules is influenced by the characteristics of the animal (species, strain, age, bacterial status) and the treatment design (dose, route of administration, duration of treatment).

In a counterpart set of experiments using conventional rats (Nugon-Baudon *et al.* 1990) PB treatment had led to the death of nearly half the animals receiving the GLS-rich diet and had enhanced greatly the deleterious effects of this feed on the growth curve and kidney weight in the surviving rats. As no GLS-linked toxic effects were found in PB-induced germ-free rats, we conclude that the influence of PB on GLS toxicity in conventional animals requires the presence of the intestinal microflora. No separate and specific interaction between the chemical inducer and GLS that would simply add its own effects to those mediated by the intestinal microflora is apparently involved.

We have confirmed that PB is an excellent inducer of hepatic cytochrome P450 and UDP glucuronosyltransferase and, to a lesser extent, of glutathione-S-transferase (Young & Lietman, 1978; Conney, 1982; Ullrich & Bock, 1984). Furthermore, our findings show that the induction level of all three enzymes is either similar (glutathione-S-transferase) or more (cytochrome P450 and UDP glucuronosyltransferase) important in germ-free rats than in conventional rats (Nugon-Baudon *et al.* 1990), although the PB dose was much lower in the former than in the latter. Indeed, germ-free rats fed on the control diet and treated with 0·1 g PB/l in drinking water for 24 d showed a threefold higher concentration of cytochrome P450 than the non-induced counterparts. Only a twofold increase had been found previously in conventional rats treated with 0·75 g PB/l for 14 d and 0·2 g PB/l for another 20 d. Similarly, the level of UDPglucuronosyltransferase activity was increased by ninefold and sixfold respectively by PB in germ-free and conventional rats. As far as glutathione S-transferase is concerned, both patterns of PB treatment led to a 1·5-fold increase in specific activity, whether intestinal microflora was present or not. These discrepancies relating to the bacterial status confirm the findings of Hietanen & Pelkonen (1979); these authors observed a higher response in several liver XME to PB induction in germ-free than in specific pathogen-free rats.

We observed that the control levels of cytochrome P450 concentration and transferase activities tend to be higher in germ-free than in conventional rats (Nugon-Baudon *et al.* 1990). This observation reinforces the conclusions by Einarsson *et al.* (1973) and Gustafsson & Persson (1975) who demonstrated an increase in the metabolism rate of steroids and barbiturates respectively that might be explained by an induced state of some XME in the absence of intestinal microflora (Einarsson *et al.* 1974).

The GLS-rich diet alone is without effect on the concentration of cytochrome P450 as well as on the specific activities of both transferases. This lack of effect is not modified by PB treatment. Once again, these phenomena are totally opposed to those observed earlier in conventional rats (Nugon-Baudon *et al.* 1990). Indeed, feeding the conventional animals with the GLS-rich diet led to a decreased cytochrome P450 concentration and a dramatic induction of both transferases.

On the whole, the findings of the present work support the idea that the activity of the intestinal microflora is at least an important step in the development of GLS toxicity, as well as in the alterations of the hepatic detoxication system induced by these xenobiotics. Subsequently, the intestinal microflora may play a key role in the protective effect of cruciferous vegetables towards chemical carcinogenesis in humans and animals.

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