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Intestinal microflora plays a crucial role in the genotoxicity of the cooked food mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)

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We investigated the impact of the intestinal microflora on the genotoxicity of 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ), a mutagenic/carcinogenic heterocyclic amine commonly found in fried meats and fish. In parallel, we also examined the effect of the microflora on the protective effect of glucotropaeolin (GT), a glucosinolate contained in cruciferous vegetables, towards IQ-induced genotoxic effect. Conventional (NF), human flora associated (HFA) and germ free (GF) rats were treated either with 90 mg/kg IQ alone, 150 mg/kg GT alone or a combination of the two by gavage and DNA damage was determined in liver and colon cells using the alkaline single cell gel electrophoresis (SCGE) or comet assay. IQ caused a significant effect in both organs of all groups. However, DNA damage was most pronounced in NF animals. In colon cells, DNA migration was 6-fold more in IQ-exposed rats as compared with untreated controls. The effect measured with liver cells was similar. In comparison to NF rats, in HFA rats, tail length of the comets was 22 and 53% lower in liver and colon cells, respectively. Significantly weaker effects were seen in GF animals (66 and 75% lower damage in hepatocytes and colonocytes, respectively, than in NF animals). Pretreatment with GT led to a complete reduction of IQ-induced DNA damage regardless of the microbial status of the animals. In addition, a moderate decrease in spontaneous DNA damage was seen in animals that received GT alone. Our results show that the microflora has a strong impact on the genotoxic effects of IQ. We conclude that the alkaline SCGE assay with rats harbouring different flora opens new possibilities to investigate the role of intestinal bacteria on health risks caused by dietary carcinogens.

Introduction

The increasing evidence for the involvement of heterocyclic aromatic amines (HAs), pyrolysis products of high protein diets, in the aetiology of human cancer (1–3) has stimulated strong efforts to identify exogenous and endogenous factors that modify health risks associated with HAs. More than 600 individual compounds and complex dietary mixtures have been studied for protective effects towards HAs (4) and several hundred articles have been published with regard to mammalian enzymes involved in the activation and detoxification of these compounds (5,6).

Data on the impact of the intestinal microflora on the genotoxic/carcinogenic effects of HAs are scarce and controversial. While on the one hand, intestinal bacteria bind HAs and thereby reduce their mutagenic effects (7,8), on the other hand, HAs are converted by representatives of the human microflora to direct-acting genotoxins (9–11) and detoxification products of HAs formed in the liver are reactivated in the gut by bacterial enzymes (6,12). Furthermore, the intestinal microflora was found to influence the activity of cytochrome P-450/AZ, enzymes involved in the activation process of HAs (13,14). To date, evidence concerning the impact of the intestinal microflora on the genotoxic effects of HAs is restricted to either *ex vivo* studies with faeces and urine of HA-exposed rats differing in microflora status or to *in vitro* mutagenicity tests in which representatives of the microflora were incubated together with individual HAs (15).

In the present study we used, for the first time, the alkaline single cell gel electrophoresis (SCGE) assay to study the influence of the type and presence/absence of the intestinal flora on DNA damage caused by the heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in rats differing in their microbial status. IQ is a potent colon carcinogen which is commonly found in fried meat, fish and chicken (16). The alkaline SCGE technique, developed by Singh *et al*. (17), is based on the migration of DNA in an electromagnetic field. This assay has been used to study genotoxic effects induced by different classes of DNA reactive carcinogens at the single cell level *in vitro* and *in vivo* (18–22). This test system has previously been employed to investigate the chemoprotective effect of dietary substances towards chemically induced DNA damage using colon cells of rats inoculated with human flora (23). In the present investigation, we used germ free (GF) rats, rats with conventional flora (NF) and germ free rats colonized with human faecal bacteria (human flora-associated rats, HFA) to measure DNA damage caused by IQ in liver and colon—target organs for tumour induction by this amine (16). The strong difference in the composition and drug metabolizing capacity between human and rodent intestinal microflora is well documented (11,24,25) and it has also been demonstrated that the enzyme activities of the human faecal flora can be simulated in rats associated with a mixed population of human intestinal bacteria (11,24,26).

We also used the same experimental model to study the effects of the gut microflora on the chemoprotective activities of glucotropaelin, a glucosinolate compound contained in *Brassica* vegetables (27), towards IQ-induced DNA damage. Glucosinolates are known to be hydrolysed by either plant or bacterial myrosinase to give rise to a variety of products which are predominantly isothiocyanates, but also include nitriles

Abbreviations: GF rats, germ free rats; GT, glucotropaeolin; HA, heterocyclic amine; HFA rats, human flora-associated rats; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; NF rats, rats with native intestinal flora; SCGE, single cell gel electrophoresis.

and thiocyanates (28–33). Isothiocyanates are well known for their chemoprotective effects against various carcinogens through their ability to affect the bioactivation, detoxification and excretion of carcinogens (31,32).

Materials and methods

Chemicals

2-Amino-3-methyl-imidazo[4,5-*f*]quinoline was purchased from Toronto Research Chemicals (Toronto, Canada). Collagenase, proteinase K, William's medium E, low melting point agarose, normal melting agarose and RPMI medium were obtained from Gibco (Paisley, UK). Inorganic salts for SCGE assays and ethidium bromide were from Merck.

Animals and treatment

Animal experiments were carried out with male Fischer 344 rats (body weight, 250 g) bred at the breeding facility of INRA in accordance with the regulations of the Animal Care Committee of INRA. Throughout the study, the animals were kept in a room that was maintained at constant temperature and humidity (24 ± 1°C, 50 ± 5%) with a 12 h light/dark cycle. Rats with their native intestinal flora (NF) were kept in standard cages (three animals/cage); germ free (GF) and human flora-associated (HFA) rats were housed (three animals/cage) in Trexler type isolators fitted with a rapid transfer system (LaCalhene, Velizy, France). To obtain HFA rats, initially germ-free animals were inoculated orally with whole faecal flora collected from a healthy adult man as described previously (29,33). The germ-free status of the rats was confirmed before inoculation with human flora, and colonization of the human flora was confirmed by microscopic examination of Gram-stained faecal material one week after inoculation.

Each treatment group consisted of three animals. The animals were deprived of feed for 24 h before being exposed to 90 mg IQ/kg body wt (0.2 ml/animal in corn oil) for 4 h, but received water *ad libitum*; negative controls received oil only. The dose level and the exposure time were chosen on the basis of results of previous experiments in which three dose levels (50, 100 and 150 mg IQ/kg body wt) and three exposure times (2, 4 and 6 h) were studied (Kassie Rabot,S., Uhl,M., Huber,W., Qin,H.-M., Helma,C., Schulte-Hermann,R. and Knasmüller, in preparation). The GF and HFA rats were exposed to IQ while still in the isolation chamber. The different groups of animals were randomized for treatment with IQ and subsequent killing as follows. Animals were numbered at the beginning of the experiment and then the numbers were written on paper sheets, and at each step of randomization sheets were put in a box and numbers consecutively drawn. In the combined treatment experiments, the rats were pretreated orally with 150 mg GT/kg in distilled water over three consecutive days. On the fourth day, the animals were exposed to IQ for 4 h, and killed. Liver and colon cells were isolated as described by Bradley *et al*. (33) and Brendler *et al*. (35), respectively. Isolated cells were resuspended in 50 and 20 ml medium, respectively, for liver and colon cells and cell viability determined with the trypan blue exclusion method (36). Cells were used in the comet assay only when cell viability was ⩾80% (37). Total cell number was in the range 9–15×10^6 hepatocytes/ml and 2–5×10^6 colonocytes/ml.

Single cell gel electrophoresis (SCGE) assay

Phosphate buffered saline, alkali (electrolysis) buffer, lysis solution, neutralization buffer and ethidium bromide stain were prepared as described by Singh *et al*. (38). Agarose coated slides were made with 1.5% normal melting agarose according to the protocol of Klaude *et al*. (39).

Microgel electrophoresis was performed according to Singh et al. (17). Briefly, 10 000 cells suspended in 90 µl 0.5% low melting point agarose were transferred to a slide pre-coated with normal melting agarose and covered with a cover slip. After allowing the low melting agarose to solidify by putting the slide on a cooled metal plate for 2 min, the cover slip was carefully removed and the slides submersed into lysis solution for 24 h. Subsequent to alkali treatment (pH 13.00, 20 min) and electrophoresis (300 mA, 25 mV, 20 min; Biometra Standard Power Pack P25), the slides were removed from the electrophoresis chamber, washed two times with neutralization buffer and stained with ethidium bromide. Analysis of DNA damage was made by measuring the comet tail lengths of the indicator cells with a fluorescence microscope (Nikon, EFD-3, 125-fold magnification) connected to a monitor with a specific macro for the NIH-public domain image analysis program (40). From each organ, three slides were prepared, and from each slide, 50 cells were analysed.

Determination of GT residues in the faeces

Glucotropaeolin residues were determined in the faeces of GF and HFA rats using the HPLC method recommended by the International Standardisation Organisation (Anonymous, 1990). All of the faeces emitted by the animals were collected once daily throughout the 3 day glucosinolate treatment (see above), weighed, freeze-dried and stored at –20°C until analysis.

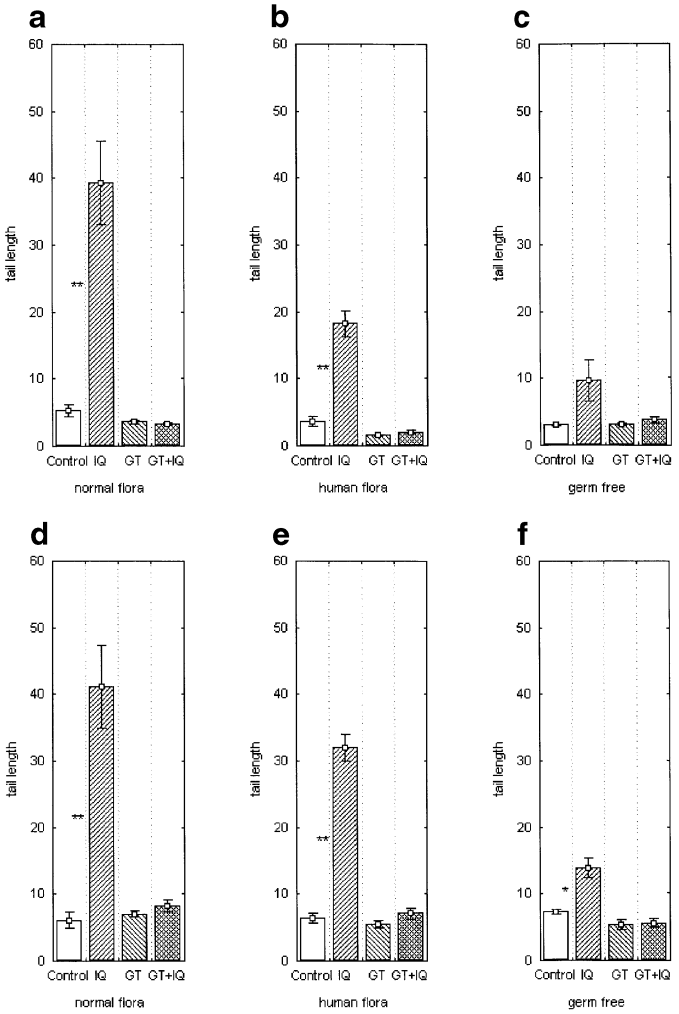

Fig. 1. Induction of DNA damage in colon cells (**a–c**) and hepatocytes (**d–f**) of GF, NF and HFA rats by IQ and its prevention by glucotropaeolin. The animals were orally treated either with 90 mg IQ/kg alone, 150 mg GT/kg alone or a combination of the two. Bars indicate means ± SEM of values measured with nine slides (three animals per treatment group, three slides per organ, 50 cells per slide). Statistical significance comparison results between experimental groups and controls are shown by ** and * for $P < 0.01$ and $P < 0.05$, respectively.

Statistics

The distribution pattern of the tail length of the different treatment groups was analysed as suggested by Tice *et al*. (41) and fitted with a γ-distribution. From each slide, the medians of the tail length were calculated, and means ± SEM and statistics were computed using results from three animals per group. Comparisons of groups of animals were done by analysis of variance based on the average value of three slides. *Post hoc* comparisons between experimental groups against control animals were done by Dunnet's test.

Results

The results of the SCGE assay with colon cells and hepatocytes are shown in Figure 1a–c and d–f, respectively. In the untreated control animals, the tail lengths of the comets were on average between 4 and 7 µm. IQ treatment caused significant DNA damage in all three groups of animals. The most pronounced induction of comets was seen in liver and colon cells of NF rats, their respective tail lengths being 6.7 and 7.3-fold longer than those seen in the corresponding controls. In HFA animals,

the IQ effect was somewhat lower (the increase in comet tail length over the background level in both organs was about 5-fold). In the colon, the mean comet tail length was significantly shorter than in NF rats. In GF animals, IQ-induced DNA damage was significant only in the liver and this effect was less than that observed in hepatocytes of other groups of rats.

Administration of GT to the different groups of rats for three consecutive days resulted in a highly significant reduction of IQ-induced DNA damage regardless of the microfloral status of the rats (Figure 1a–c and d–f). The comet tail length of hepatocytes and colonocytes from animals treated with IQ and GT was almost similar to that measured in cells from the control animals. The effect of GT on the background level of DNA damage was not significant in any of the three control groups although moderate decreases were observed.

Analysis of the faecal GT contents of GF, HFA and NF rats showed that the compound was not detectable in the latter two groups. In the GF animals, 11–14 μmol (5–6 mg) GT/g faeces were found at each collection time; on the whole, 21–44 μmol (9–19 mg) GT/rat were recovered daily in the faeces, which accounted for 25–50% of the daily ingested dose.

Discussion

The results of the present study show the role played by the intestinal microflora in modulating IQ-induced DNA damage in rat liver and colon tissues, which are targets for tumour induction by this heterocyclic aromatic amine. The SCGE technique was found to be an ideal approach for such investigations as it is fast and easy to perform and enables the measurement of DNA damage in different inner organs. However, one of the disadvantages of this model is that the dose of IQ required to induce significant effects is substantially higher than human exposure levels. This is probably due to the lower activation capacity of rats for HAs compared with humans (42). Nevertheless, we anticipate that this model enables investigation of the impact of exogenous and endogenous factors on the health risks of HAs. In a recently carried out experiment, we found that reduction of IQ-induced DNA damage in liver and colon by *Brassica* vegetables is paralleled by a decrease in the formation of pre-neoplastic lesions in the colon and liver (Kassie et al., in preparation).

As described above, the extent of DNA migration in animals harbouring intestinal microrflora was, depending on the type of microflora and on the organ, 3–5-fold higher than that seen in GF rats. This observation can be taken as an indication that the health risks of IQ, and possibly other heterocyclic amines as well, are strongly influenced by the intestinal microflora. Hitherto, research on the bioactivation and detoxification of HAs has focused predominantly on mammalian enzymes and very little data are available on the role of intestinal flora on the metabolism of HAs (15). In two studies (43,44) where urinary and faecal excretion of Trp-P-1 and DiMeIQx was compared in GF and NF rats, the role of the intestinal flora in the metabolism of these compounds was found to be minor. Some *in vitro* studies show that certain intestinal microbes, such as Lactobacilli, inactivate heterocyclic amines (7,8) whereas incubation of these compounds with other intestinal strains led to an increase in the bacterial mutagenicity of the amines (9–11). If and to what extent these findings can be extrapolated to the *in vivo* situation is largely unknown.

Three different mechanisms might account for the much higher DNA migration seen in rats harbouring microflora as compared with GF rats. (i) Cleavage of IQ–glucuronide conjugates in the gut by bacterial enzymes. Although it is well known that glucuronidation is a major detoxification pathway for heterocyclic amines in man and rodents (45,46), it is not clearly known if the conjugation products are split by enzymes of the intestinal bacteria. Alexander *et al*. (47) reported cleavage of PhIP–glucuronide conjugates by bacterial enzymes. On the other hand, incubation of the urine of HA-treated laboratory animals with bacterial β-glucuronidase failed to result in enhanced bacterial mutagenicity (48). (ii) Another possible explanation is the conversion of IQ to 7-OHIQ, a potent direct acting mutagen, by representatives of the intestinal microflora (10,11). However, it is unlikely that this metabolite accounts for the present findings as subsequent experiments showed that 7-OHIQ is devoid of mutagenic activity in mammalian cells and does not induce tumours in mice and rats (49). Nevertheless, other hitherto unknown activation reactions might take place. Recently, we found that incubation of IQ and meat extracts with *Bacteriodes fragilis*, an abundant microfloral species, leads to a strong increase in bacterial mutagenicity of IQ. These effects were seen only after addition of liver enzyme mix and this indicates that metabolites other than the hydroxy-derivative are involved (15). In this context, it is notable that high concentrations of Bacteriodes species in humans were found to be associated with an increased risk for colon cancer in humans (50). (iii) Finally, indirect mechanisms might also play a role: Övervik *et al*. (13) found a higher faecal excretion of meat derived mutagens in NF rats than in GF rats. This difference in the excretion pattern was paralleled by higher hepatic EROD activity, an enzyme catalysing the first step of activation of HAs (15). These observations suggest that intestinal bacteria might modulate the metabolism of HAs via induction of xenobiotic drug metabolizing enzymes.

The results of the combined treatment experiments in which the animals received GT before administration of the carcinogen show that the glucosinolate is highly protective towards IQ-induced DNA damage. The observation of a strong protective effect in the GF animals was quite unexpected as it is assumed that the chemoprotective effects of *Brassica* vegetables are caused by isothiocyanates and indoles which are breakdown products of glucosinolates and are formed either by plant enzyme or by intestinal bacteria in the colon (27,33). However, there is also a report which indicates spontaneous degradation of glucosinolates in the upper digestive tract (51).

This is the first report in which the SCGE assay was used with rats differing in their intestinal microflora to investigate the complex interactions between intestinal bacteria, chemical carcinogens and chemoprotective compounds. Our findings show that the intestinal flora has a strong impact on the genotoxic effects of IQ which, in the past, has been totally underestimated probably due to lack of an appropriate experimental system.

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