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The multiple intestinal neoplasia (Min) mice have a mutation in the murine adenomatous polyposis coli (Apc) gene rendering them highly susceptible to spontaneous intestinal adenoma formation, similar to the familial adenomatous polyposis (FAP) syndrome in humans. We studied whether the most abundant mutagenic heterocyclic amine isolated from cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), could influence early intestinal neoplasia which often includes a diet consisting of high consumption of well-done red meat. Highly mutagenic and carcinogenic heterocyclic amines isolated from the crust of fried meat are the homolog of the human APC gene (6). These mice are highly susceptible to spontaneous formation of numerous tumors both in the large and small intestine, most of which are adenomas with some areas of carcinoma in situ in older mice (5). The C57BL/6J-Min/+ mouse presents an opportunity to study pathogenesis of a neoplasia in which the initial molecular defect is the same between human and mouse. Therefore, in spite of the not totally equivalent distribution of tumors between the Min mice and the FAP patients, the Min model is a valuable one for the study of FAP. In addition, this model is useful for the study of sporadic colorectal and small intestinal neoplasms as well.

Colorectal cancer appears to be linked to a Western life style, which often includes a diet consisting of high consumption of well-done red meat. Highly mutagenic and carcinogenic heterocyclic amines isolated from the crust of fried meat have therefore been suggested as candidate human colorectal carcinogens (7). The heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) was first isolated from fried ground beef (8), and is the most abundant heterocyclic amine in various cooked meats and fish (7,8). Although its mutagenic potency in the Salmonella typhimurium assay is relatively low (7), PhIP is more mutagenic than other heterocyclic amines in cultured mammalian cells (9,10). PhIP induced colon tumors in male, and predominantly mammary tumors in female F344 rats (11). In Nagase analbuminemic male rats PhIP induced tumors in the small intestine, but lymphomas in both sexes (14). However, low numbers of ACF have been reported in female C57BL/6J mice treated with PhIP (15). Recently, PhIP was shown to increase the size of intestinal polyps in ApcΔ716 knockout mice (16).

It appears that intestinal tumor development both in humans (2,17) and Min mice (17,18) is associated with loss of function of both APC alleles. We therefore hypothesized that the Min/+ mice, having already lost one APC allele, would be particularly susceptible to intestinal carcinogens that preferentially affect the APC gene, compared with +/+ mice. Recently, it was reported that PhIP had induced a specific and unique mutation in the rat Apc gene, changing a 5’-GGGA-3’ sequence to 5’-GGGA-3’, in colon tumors in F344 rats (19). The main objectives of this work were to examine whether C57BL/6J-Min/+ mice are suitable as a sensitive short-term animal model for carcinogenicity studies, and to use this model to study the effect of PhIP on intestinal neoplasia.

The experiment was terminated after 10 weeks. It was thought that this would allow enough time for induction of ACF (13,15), as well as for the growth of a substantial number of spontaneous tumors (5), in the intestinal tract of the C57BL/6J-Min/+ mice. As a comparison, other mice with both wild-type alleles present (C57BL/6J-+/+) were treated with PhIP. The end points used were number and size of intestinal tumors, cystic crypts and ACF.
after exposure to ENU (25). The lesions described in these two studies (24,25) of the smallest tumors, with diameter 0.1–1.0 mm, was found and dATP (Promega Corp., Madison, WI), 2.5 mM MgCl₂, 50 mM Tris–HCl seem to be identical. In this study, we identified lesions according to the after PhIP exposure (Apc recombination in mouse embryonic stem cells (24). These lesions were found PhIP treatment increased the number of the smallest tumors mostly in the small intestine and had lost the wild-type Min PhIP treatment Tumors in the small intestine E. Merck (Darmstadt, Germany) if not stated otherwise. treatment.

Chemical Co.). The reagents were purchased from Sigma Chemical Co. or body weight were found with regard to either genotype or

Statistical analysis

The results were analyzed for differences between the two treatments (PhIP or vehicle) by Student’s t-test or the non-parametric Mann–Whitney rank sum test, as found appropriate (SigmaStat software, Jandel Scientific, Germany). In addition, the Fischer exact probability test (two-tailed probability) was used to evaluate incidence data. A P-value of <0.05 was considered significant.

Results

Animal growth

The male Min/+ mice showed ~10% lower body weight compared with the +/+ mice at the termination of the experiment, but no effect of PhIP treatment was seen in either group (data not shown). In the female mice, no difference in body weight were found with regard to either genotype or treatment.

Tumors in the small intestine

In Min/+ mice, the incidence of tumors in the small intestine was 100% both in females and males that received PhIP or vehicle (Table I), whereas in +/+ mice no tumors were found in the small intestines irrespective of sex or treatment. In Min/+ males, exposure to PhIP induced a significant increase in the number of tumors in the proximal section of the small intestine compared with Min/+ males given the vehicle (Table I). Treatment with PhIP resulted in a somewhat increased number of tumors in the middle and distal sections of the small intestine, and an increase in total number of tumors in the organ, but this did not reach statistical significance. There was no significant difference in tumor number in either section of the small intestine in female mice that were exposed to PhIP (Table I).

When grouped according to size, it became apparent that PhIP treatment increased the number of the smallest tumors in the proximal section of the small intestine (Figure 1). In male Min/+ mice (Figure 1a), a significantly higher number of the smallest tumors, with diameter 0.1–1.0 mm, was found after PhIP exposure (P < 0.02, Student’s t-test). The same tendency was seen in the female Min/+ mice (Figure 1b), but this was not statistically significant.

PhIP treatment did not increase the size of the tumors in

was divided into three sections of equal length, namely the proximal, middle and distal sections, while the large intestine was not further divided. The intestinal tissues were fixed flat between filter paper for at least 48 h in 10% neutral buffered (28.9 mM NaH₂PO₄·H₂O, 44.4 mM Na₂HPO₄·2H₂O) formalin prior to 3 min staining with 0.2% methylene blue (George T Garr Ltd, UK) dissolved in the same formalin solution. The tissues were examined under a light microscope at a magnification of ×20 or ×40, to score tumors in both the small and large intestine, the cystic crypts of the small intestine, and the ACF of the large intestine. The diameters of the tumors and cystic crypts in the length direction of the intestines were defined by an eyepiece graticule, and given in units of mm after correcting for the magnification used.

The incidence was defined as the number of mice with tumors or cystic crypts/number of mice in the group. Both number and diameter of tumors and cystic crypts were recorded. The results are given as means of the treatment groups ± SD, for the numbers of tumors and cystic crypts, while the diameter of the tumors in the large intestine is given as the mean of the tumors present in each group ± SD.

Histological evaluation

Fixed tissue samples were embedded in paraffin and sectioned at 4-μm thickness with a Reichert-Jung slide microtome, and stained with hematoxylin-eosin-saffron.

Materials and methods

Breeding of mice

The mice were bred at the National Institute of Public Health, Oslo, Norway, from the pedigrees originally purchased from The Jackson Laboratory (Bar Harbor, ME). The Min pedigree was maintained by mating C57BL/6J-+/+(wild-type) females with C57BL/6J-Min+/- males. The Min mutation cannot be propagated in the female, because anemia and intestinal adenomas interfere with pregnancy (5). All mice used in the experiment were related within the number of generations (<12) necessary for securing their status as inbred.

Genotype analysis

The Min/+ mice produced were identified by allele-specific polymerase chain reaction (PCR) (20,21). DNA was isolated from blood according to the procedure obtained from The Jackson Laboratory. Approximately 40 μl blood was collected from the saphenous vein on the hind leg of the mice in a heparinized microhematocrit tube (Vitrex, Modulohim A/S, Herlev, Denmark) and transferred to 70 μl of a phosphate buffered saline (PBS)/EDTA solution (1 mM EDTA, 137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ in a microcentrifuge tube on ice, and mixed by inversion. After centrifugation for 1 min at 13 000 g, the supernatant was discarded and the pellet was resuspended by vortexing in 70 μl of a blood wash solution [0.32 M sucrose, 10 mM Tris–HCl (pH 7.5), 5 mM MgCl₂, 1% Triton X-100]. This washing step was repeated twice to remove all hemoglobin. After centrifugation the supernatant was discarded and the pellet was resuspended by vortexing in 75 μl PCR digestion buffer (10 mM Tris–HCl (pH 8.3), 2.5 mM MgCl₂, 125 mM NaCl, 100 mM KCl, 0.1 mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20 (Koch-Light Laboratories, Ltd, Berks, UK)]. Aliquots of 2 μl of 10 mg/ml proteinase K (Sigma Chemical Co., St Louis, MO) were added before incubation at 55°C for 1 h. Finally the samples were incubated at 95°C for 10 min to inactivate the proteinase. The samples were stored at −20°C until PCR amplification. The PCR reaction was carried out with a 1605 Air Thermocycler (Idaho Technology, Inc., Idaho Falls, ID) as follows. Genomic DNA (5 μl of a 1:10 dilution of the isolated DNA solution) was amplified in a 10-μl reaction volume per sample, which contained final concentrations of 1.0 μM MAPC M, 125 μM MAPC 9 (primers [21], purchased from R&D Systems Europe, Ltd, Abingdon, UK), 200 μM each of dCTP, dGTP, dTTP and dATP (Promega Corp., Madison, WI), 2.5 mM MgCl₂, 50 mM Tris–HCl (pH 8.3), 0.25 mg/ml bovine serum albumin (BSA), 0.015 U/μl of Taq polymerase (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). The amplification conditions were 1 min at 94°C before 35 cycles at 94°C for 5 s, 54°C for 10 s and 74°C for 30 s, followed by a final extension at 74°C for 2 min. The PCR products were visualized by electrophoresis through 2% agarose (Promega Corp.), followed by staining with ethidium bromide (Sigma Chemical Co.). The reagents were purchased from Sigma Chemical Co. of E. Merck (Darmstadt, Germany) if not stated otherwise.

PhIP treatment

Four- to 7-week-old Min/+ and +/+ mice of both sexes were injected intraperitoneally (i.p.) with either 50 mg/kg PhIP (a generous gift from Dr Errol Zeiger, Research Triangle Park, NC) dissolved in equal parts of dimethylsulfoxide (DMSO) (Aldrich Chemical Co., Germany) and 0.9% NaCl, or the same volume of vehicle, once a week for 4 weeks. PhIP was given as i.p. injections for practical and economical reasons, rather than in the food, which is the most relevant route of exposure. PhIP is rapidly absorbed and distributed in mice by i.p. as well as by Peroral (p.o.) administration (22). The same metabolites of PhIP are formed (22), and PhIP is shown to be a potent intestinal mutagen (23), given both by routes of administration. The mice were housed in polystyrol cages in a room with a 12-h light/dark cycle and controlled humidity and temperature, and were given a standard diet (B&K Universal Ltd, N.Humberside, UK) and water ad libitum. The mice were killed by cervical dislocation 10 weeks after start of the experiment.

Scoring of tumors, cystic crypts and ACF

Nascent intestinal polyps consisting of microadenoma covered with a layer of normal villous epithelium were recently described in the small intestines of mice containing a mutant Apc gene (ApcΔ716) constructed by homologous recombination in mouse embryonic stem cells (24). These lesions were found mostly in the small intestine and had lost the wild-type Apc allele, whereas the mutant allele remained unchanged. A distinct type of intestinal lesion, called cystic crypts, was also described in the small intestines of Min mice after exposure to ENU (25). The lesions described in these two studies (24,25) seem to be identical. In this study, we identified lesions according to the descriptions in References (24,25), using the same cystic crypts.

The small and large intestines were removed separately, rinsed in ice-cold PBS, 15 μl 15 mM NaCl, 13 mM KH₂PO₄, 53 mM NaHCO₃, 2.7 mM CaCl₂·2H₂O, 0.14 mM NaCl, pH 7.4) and slit open along their longitudinal median axis. The small intestine
Table I. Number of tumors in the small intestines of C57BL/6J-Min/+ mice treated with PhIP or vehicle

<table>
<thead>
<tr>
<th>Sex</th>
<th>Genotype – Treatment</th>
<th>Incidencea</th>
<th>Proximal sectionb</th>
<th>Middle sectionb</th>
<th>Distal sectiond</th>
<th>Totalc</th>
</tr>
</thead>
<tbody>
<tr>
<td>♀</td>
<td>Min/+ – vehicle</td>
<td>7/7</td>
<td>8.1 ± 4.5</td>
<td>30.1 ± 14.0</td>
<td>40.4 ± 20.2</td>
<td>78.7 ± 34.1</td>
</tr>
<tr>
<td></td>
<td>Min/+ – PhIP</td>
<td>7/7</td>
<td>10.9 ± 7.9</td>
<td>23.1 ± 15.2</td>
<td>37.0 ± 27.5</td>
<td>71.0 ± 49.9</td>
</tr>
<tr>
<td>♂</td>
<td>Min/+ – vehicle</td>
<td>6/6</td>
<td>6.0 ± 4.6</td>
<td>22.2 ± 13.0</td>
<td>36.3 ± 22.5</td>
<td>64.5 ± 37.9</td>
</tr>
<tr>
<td></td>
<td>Min/+ – PhIP</td>
<td>6/6</td>
<td>15.3 ± 5.5</td>
<td>26.5 ± 14.2</td>
<td>39.7 ± 21.6</td>
<td>81.5 ± 38.2</td>
</tr>
</tbody>
</table>

aNumber of mice with tumors/number of mice in the group.
b,c,dNumber of tumors (group mean ± SD) in the proximal, middle and distal sections of the small intestine, respectively.
cTotal number of tumors (group mean ± SD) in the whole small intestine.
dSignificantly higher number of tumors with PhIP compared with vehicle (P = 0.015, Mann–Whitney rank sum test).

Fig. 1. Number of tumors (mean ± SD) of various sizes in the proximal section of the small intestine of (a) male and (b) female Min/+ mice treated with PhIP (●) or vehicle (○). Significantly higher numbers of tumors were found with PhIP compared with vehicle (t-test).

any section of the small intestine, either in male or female Min/+ mice (data not shown).

Histological evaluation revealed that the tumors in the small intestine were adenomas with no evidence of carcinoma (data not shown). The tumors from Min/+ mice exposed to PhIP were not histologically distinguishable from the tumors from Min/+ mice exposed to vehicle (data not shown).

Cystic crypts in the small intestine

In Min/+ mice, the incidences of cystic crypts in the small intestine were 100% and 83.3% in males, and 71.4% and 85.7% in females, treated with PhIP and vehicle, respectively (Table II), whereas in +/+ mice no cystic crypts were found in the small intestines irrespective of sex or treatment. Most of the cystic crypts were found in the proximal section of the small intestine, and none in the distal section, in either sex. In male Min/+ mice, a significantly higher number of cystic crypts was found after treatment with PhIP, compared with vehicle, in the proximal section of the small intestine (Table II). A tendency for increased numbers of cystic crypts after treatment with PhIP was also found in the middle section of the small intestine, as well as an increase in total number of cystic crypts in this organ, but this did not reach statistical significance. In female mice, no significant effect of PhIP on number of cystic crypts was found in either section of the small intestine (Table II).

The cystic crypts ranged in size from 0.15 to 0.50 mm in diameter. No effect of PhIP were found on the size of the cystic crypts (data not shown).

Tumors in the large intestine

Few tumors were found in Min/+ mice, either treated with PhIP or vehicle, and no apparent effects of PhIP were observed on number or size of tumors in either sex (Table III). No tumors were found in the large intestines of +/+ mice irrespective of sex or treatment.

ACF in the large intestine

After PhIP exposure, the incidences of ACF were 100% in male and 85.7% in female Min/+ mice, and this was significantly higher than in the vehicle exposed mice, for both sexes (Table IV). In one vehicle exposed male Min/+ mouse, one ACF with three AC was found, while no ACF was found in vehicle exposed female Min/+ mice. In Min/+ mice, PhIP induced significantly higher numbers of ACF and AC compared with vehicle in both males and females (Table IV). No ACF were found in the large intestines of male +/+ mice receiving either PhIP or vehicle, or in females exposed to vehicle (Table IV). In one female +/+ mouse exposed to PhIP, one ACF consisting of three AC was observed. Min/+ mice exposed to PhIP had significantly higher values than +/+ mice exposed to PhIP, in both parameters, and in both males and females (Table IV).

Other findings

One female Min/+ mouse exposed to vehicle had a histologically verified epidermoid cyst (1.3 cm in diameter) located at the side of the chest. No such lesions were found in Min/+ mice exposed to PhIP, or in any +/+ mice.

Discussion

The major findings in this study are that exposure to PhIP increased the number of small tumors (Table I) and cystic crypts (Table II) in the proximal section of the small intestine in male Min/+ mice, and the number of ACF in the large
PhIP exposure in one female mouse (Table IV). In mice exposed to PhIP or vehicle. They also had the same size of the tumors in any of the three sections of the small intestine, respectively. The cystic crypts induced by PhIP seemed to be similar to the intestinal tumors, an initiating effect after exposure to PhIP (P = 0.03, Mann–Whitney rank sum test). Small intestine. Therefore, exposure to PhIP appears to act early in the neoplastic process. However, if the experiment had been continued for longer, an effect of PhIP would have had time to grow to a larger size.

PhIP increased the number of cystic crypts in the proximal section of the small intestine in male Min/+ mice (Figure 1a). Apparently, PhIP did not promote the growth of pre-formed tumors as it had no effect on the mean size of the tumors in any of the three sections of the small intestine. Therefore, exposure to PhIP appears to act early in the neoplastic process. However, if the experiment had been continued for longer, an effect of PhIP would have had time to grow to a larger size.

PhIP increased the number of cystic crypts in the proximal section of the small intestine in male Min/+ mice (Table II). The cryptic crypts induced by PhIP seemed to be similar to the lesions found in the ApcΔ716 mice (24), and in Min mice exposed to ENU (25). They were found only in the small intestine, most predominantly in the proximal end, in Min/+ mice exposed to PhIP or vehicle. They also had the same size (0.15–0.50 mm in diameter). None were found in the PhIP exposed Min/+ mice. However, they initiated few tumors in this strain. Hence, this finding further supports the hypothesis that exposure to PhIP has an initiating effect in this model.

Exposure to PhIP could also clearly affect the neoplastic process in the large intestine of Min/+ mice, as this exposure increased the number of ACF and the number of AC in both intestines of both male and female Min/+ mice (Table IV). In contrast, no tumors or cystic crypts were found in +/+ mice either exposed to PhIP or vehicle, and only one ACF was observed after PhIP exposure in one female +/+ mouse. These results clearly show that Min/+ mice are more sensitive to PhIP than +/+ mice, and as such are useful as an animal model for carcinogenicity studies. This susceptibility to an intestinal carcinogen is in accordance with earlier experiments showing that Min/+ mice were more sensitive to the induction of mammary carcinomas (27) and intestinal tumors and cystic crypts (25) upon exposure to ENU, than +/+ mice.

The absence of tumors and cystic crypts, and the very small number of ACF, in the intestinal tract of +/+ mice observed after PhIP exposure, are in accordance with earlier experiments with other non-mutated mice. No tumors were found in the gastrointestinal tract of CDF1 mice given a diet with 0.04% PhIP for 579 days, which induced lymphomas in both sexes (14). PhIP (total dose 150 mg/kg) given by gavage twice at 4 days apart only induced very low numbers of ACF in female CF1 mice (15). This weak carcinogenic effect of exposure to PhIP in the intestinal tract of +/+ mice is probably not caused by non-mutability of the crypt stem cell population in this organ, because PhIP has been shown to be a potent mutagen in the small intestine of mice (23). However, in male Min/+ mice, exposure to PhIP increased the number of cystic crypts by six times and tumors 2.6 times, which was in the same order of magnitude as the nine times increase in mutagenic effect (23).

In this animal model with a continuous formation of new intestinal tumors, an initiating effect after exposure to PhIP on mice exposed for 4 weeks at 4–7 weeks of age and killed 10 weeks later, would only be expected to lead to an increase in the number of the smallest tumors. We found that PhIP only increased the number of the smallest tumors, with diameter 0.1–1.0 mm, in the proximal section of the small intestine in male Min/+ mice (Figure 1a). Apparently, PhIP did not promote the growth of pre-formed tumors as it had no effect on the mean size of the tumors in any of the three sections of the small intestine.
sexes (Table IV). The number of tumors found in the large intestine of Min+/− mice is usually lower than in the small intestine (5). The fact that PhIP increased the number of preneoplastic lesions, ACF and AC (Table IV), but had no effect on the number or size of the tumors in the large intestine (Table III), indicates that the process of tumor development in the large intestine is slower than in the small intestine. Thus, there is consistency between these findings in the large intestine and the results from the small intestine where PhIP had no effect on the larger tumors, but only on the smallest ones (Figure 1) and on the cystic crypts (Table II). An experiment of longer duration would clarify whether PhIP might also affect tumor development in the large intestine.

Epidermoid cysts are common extracolonic manifestations in FAP patients (28). One such lesion was found in a Min+/− mouse treated with vehicle, and none in mice exposed to PhIP, in our study. A high incidence of such lesions was reported in Min+/− mice after ENU exposure (25), which is in contrast to our results with PhIP.

PhIP only significantly increased the number of tumors (Table I) and cystic crypts (Table II) in the most proximal section of the small intestine. In Min+/− mice with spontaneous tumors, there is a tendency for higher numbers of tumors in duodenum and proximal ileum compared with the distal ileum in most mice, although the inter-individual variation is large (5). The reason for the regional difference in the manifestation of an inherited genetic lesion and susceptibility to PhIP exposure, is not known. No difference in mutability was found between the proximal and distal section of the small intestine of mice exposed to PhIP in one study (23). However, there may be regional differences along the intestinal tract in expression of various endogenous genes, e.g. genes coding for metabolic enzymes, in the differentiated cell types. This regional pattern is shown to be preserved in tumors from various positions along this axis in the Min+/− mice (29).

The effects after exposure to PhIP on the numbers of tumors, cystic crypts and ACF were more pronounced in male than in the female Min+/+ mice, although no significant differences were found between the sexes. These results are in accordance with an experiment where N-OH-PhIP injected i.p. for 5 consecutive days was reported to increase the number of intestinal polyps by three times in male ApcΔ716 heterozygous knockout mice only (16). In F344 rats, PhIP induced colon tumors in males, but predominantly mammary tumors in the females (11). This sex difference in the rats was not caused by a difference in DNA adduct levels, but an enhanced cell proliferation and also ACF formation was seen in the males compared to the females (30). In our study, there was a tendency for higher numbers of ACF and AC in males compared with females (Table IV). Whether a difference in cell proliferation is also found between the sexes in the Min+/− mice, should be examined.

In contrast to our study where exposure to PhIP resulted in an increased number, but not size of tumors in the proximal section of the small intestine in male Min+/+ mice, lower doses of PhIP given in the feed changed the distribution of intestinal polyps (from the small and large intestines together) of ApcΔ716 knockout mice to a larger size range (16). However, the number of polyps was also increased in this study, but did not reach statistical significance, probably because of the small number of male mice in the control group.

Min+/+ mice have a germline nonsense mutation in codon 850 of the Apc gene, changing a leucine (TTG) to a stop (TAG) codon (6). Inactivation of the remaining allele of the Apc gene is found in 100% of the studied spontaneous intestinal adenomas from Min+/+ mice (17,18). We do not yet know what the effect of exposure to PhIP on the Min+/− mice is. Neither can it be excluded that the effect of PhIP is to induce mutations in other genes relevant for intestinal carcinogenesis, such as Mcc, Dcc, p53, K-ras and Mom-1.

The concentrations of PhIP in various cooked foods have been reported to be in the range of 0.5–70 ng/g (7). Most of the long-term animal carcinogenicity studies with PhIP have used doses of ~400 mg/kg diet (11,12,14), giving a total dose of ~48 mg after 4 weeks exposure in mice. In this study, the mice received total doses of ~4–6 mg PhIP in the course of 4 weeks. However, even if these neoplastic effects of PhIP were attained by using high doses, they still point to the importance of further studies with PhIP and other food mutagens for the etiology of human cancers. PhIP is particularly important, because it is the most abundant heterocyclic amine, and its carcinogenic organotropism overlaps with the types of neoplasia most commonly observed in Western countries.

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