Intestinal Tumorigenesis in Multiple Intestinal Neoplasia Mice Induced by the Food Mutagen 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine: Perinatal Susceptibility, Regional Variation, and Correlation with DNA Adducts

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ABSTRACT

In our previous experiments, multiple injections with the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) were used to induce intestinal tumors in C57BL/6J-multiple intestinal neoplasia (Min+)/+ mice. To define the period of highest susceptibility to PhIP perinatally, we first determined the effect of a single s.c. injection. Ten or 50 mg/kg PhIP increased the number and diameter of small intestinal tumors dose-dependently in 3-day-old Min/+ mice. In the colon, only 50 mg/kg PhIP increased the incidence and number of tumors. The number of dysplastic aberrant crypt foci decreased from weeks 7 to 11. In the same period, an increase in the number of tumors was seen, indicating that over time the dysplastic aberrant crypt foci develop into tumors. Min/+ mice were then exposed in utero through their dams being given one s.c. injection of 50 mg/kg PhIP 3 days before giving birth or were exposed directly to the same dose on day 3, 12, or 36 after birth. Remarkably, the most susceptible period for tumorigenesis in the small intestine was between days 3–12 after birth, whereas in the colon it was from day 3 before to day 3 after birth. Furthermore, we examined whether the formation of DNA adducts determined after 24 h could explain the observed time-dependent and regional susceptibility to PhIP. A higher level of PhIP-DNA adducts was found after exposure on day 12 after birth, compared with day 36 after birth, in all parts of the small intestine but not in the colon, which was in close accordance with the numbers of tumors present. The levels of PhIP-DNA adducts along the intestines were highest in the middle and distal parts of the small intestine, where tumor numbers were also the highest. In conclusion, Min/+ mice are most susceptible to intestinal tumor induction by PhIP from day 3 before to day 12 after birth, and this susceptibility could at least partly be explained by the formation of PhIP-DNA adducts.

INTRODUCTION

Colorectal cancer appears to be associated with a high consumption of red meat (1), especially when cooked well done (2). Highly mutagenic and carcinogenic heterocyclic amines isolated from fried ground beef (4) and is the most abundant heterocyclic amine in various cooked fish and meats (3, 4). In cultured mammalian cells, PhIP is more genotoxic than the other heterocyclic amines (5, 6).

To be mutagenic, genotoxic, and carcinogenic, PhIP must be metabolized to a reactive ultimate metabolite (7, 8). The initial activation step is N-hydroxylation which is catalyzed predominantly by hepatic cytochrome P-450 1A2 (CYP1A2; Refs. 9 and 10). Whether conjugated to a glucuronide in the liver (11) and transported via the bile to the colon where bacterial β-glucuronidases cleave, particularly the N3-glucuronides to regenerate N-hydroxyarylamines, or transported as stable N-hydroxy- or N-acetoxy-arylamines via the circulation to peripheral tissues including the colon (12), once reabsorbed into the colonic mucosa N-hydroxyarylamines can be further activated through O-acetylation, catalyzed by N-acetyltransferases (13), or sulfonation, catalyzed by sulfotransferases (14). The N-acetoxy or N-sulfoarylxy ester derivatives formed are unstable and spontaneously hydrolyze to a reactive electrophilic arylnitrenium ion. This ultimate metabolite is able to bind covalently to DNA and other macromolecules, forming adducts that may cause mutations and lead to induction of cancer (15). In rats, PhIP induces tumors in the colon, small intestine, and cecum, as well as in the mammary glands in females (16–18) and the prostate in males (19), and also colonic ACF (18, 20). In mice, however, PhIP induces lymphomas and no intestinal tumors (21), although PhIP-induced ACF have been reported (22).

C57BL/6J-Min/+ mice are heterozygous for a germline nonsense mutation in codon 850 of the tumor suppressor gene Apc, changing a leucine (TTG) to a stop (TAG) codon and thereby producing a truncated nonfunctional Apc protein (23, 24). Multiple small intestinal adenomas, as well as more sporadic colonic adenomas, develop spontaneously in all of these mice, and some of these progress to adenocarcinomas (23, 24). The Min/+ mouse is an excellent model for the dominantly inherited autosomal disorder FAP, characterized by an early development of multiple colorectal adenomas, as well as for sporadic colorectal cancer, both conditions being caused by various mutations in the human APC gene (25–28). It appears that a loss of function of both Apc/ APC alleles is an early event in intestinal tumor development in both mice (29–32) and humans (28, 30). Accordingly, the murine FAP models are particularly susceptible to intestinal carcinogens that affect the Apc gene.

We have reported previously that adult Min/+ mice were more susceptible to four weekly i.p. injections of 50 mg/kg PhIP than wild-type mice (33). However, this effect was restricted to a slight increase in the number of nascent tumors in the proximal small intestine of male mice. In accordance with these results, PhIP exposure beginning at 4 weeks of age caused no (34) or only a moderate (35) tumorigenic effect in other murine FAP models with one mutated Apc allele. The results from our previous study (33) indicated that spontaneous tumor initiation in Min/+ mice took place much earlier than the age at the start of PhIP exposure (4—7 weeks of age). More recently, we have shown that Min/+ mice are much more susceptible to PhIP if exposed neonatally (36) than as young adults (33). When neonatal (3–6 days old) Min/+ mice were given 8 s.c. injections (3
INTESTINAL TUMORIGENESIS

MATERIALS AND METHODS

The main objective of this study was to define more closely the period of susceptibility to PhIP perinatally to optimize the use of this mouse model, as well as learn more about PhIP-induced intestinal tumorigenesis. Initially, we examined the dose necessary to induce intestinal tumors when giving only a single s.c. injection of PhIP. We also examined whether a single dose of PhIP via breast milk could induce tumors. Furthermore, we investigated whether PhIP-DNA adduct levels correlated with the susceptibility to intestinal tumor induction in Min+/ mice exposed to PhIP at different time points after birth and with the observed regional variation in susceptibility along the intestines.

Mouse Breeding. The mice were bred at the National Institute of Public Health, Oslo, Norway, from mice originally purchased from The Jackson Laboratory (Bar Harbor, ME). The Min pedigree was maintained by mating C57BL/6j Min+/ (wild-type) females with C57BL/6j Min+/ males. The Min mutation was propagated through the males, because anemia and intestinal adenomas interfere with pregnancy (23). All mice used in the experiments were related within the number of generations (<12) necessary for securing their status as inbred. The Min+/ mice used in this study were identified by allele-specific polymerase chain reaction, as described in detail previously (33), using DNA isolated from blood drawn after the lactation period. The mice were housed in plastic cages in a room with a 12-h light/dark cycle and controlled humidity (55 ± 5%) and temperature (20–24°C). Water and diet were given ad libitum. The mice were given a breeding diet, SDS RM3 (E), from Special Diets Services (Witham, United Kingdom) during gestation and until 5 weeks of age, and a standard maintenance diet (B&K Universal, Grimston, United Kingdom), thereafter.

PhIP. PhIP of ≥98% purity purchased from Toronto Research Chemicals (North York, Ontario, Canada) was dissolved in concentrated HCl, which thereafter was evaporated. The PhIP-HCl was dissolved in 0.9% saline, and the pH was adjusted to 3.5.

Induction and Scoring of Tumors and Dysplastic ACF. Three (3–5)-day-old Min+/ pups of both sexes were given a single s.c. injection of either the same dose as in the previous studies, 50 mg/kg, or lower doses, 10 and 1 mg/kg PhIP. These mice were terminated by cervical dislocation at 7 (10 or 50 mg/kg PhIP) or 11 (1, 10, or 50 mg/kg PhIP) weeks of age. Untreated control mice were also terminated at 7 or 11 weeks of age. To define more closely the critical period of PhIP exposure perinatally, Min+/ pups were either exposed to PhIP in utero, the dams receiving a single s.c. injection of 50 mg/kg PhIP on day 3 (1–4) before birth, or were given a single injection of 50 mg/kg PhIP either on day 3 (3–5), 12 (12–13), or 36 (35–37) after birth. Another group of pups was exposed through breast milk from dams given a single s.c. injection of 50 mg/kg PhIP on day 3 (3–4) after giving birth. All mice were terminated at 11 weeks of age.

The colon and small intestine were removed separately, rinsed in ice-cold PBS [1.14 mM NaH2PO4, 5.53 mM Na2HPO4, 12H2O, and 0.14 M NaCl (pH 7.4)], and slit open along the longitudinal axis. The intestinal tissues were then spread flat between sheets of filter paper and fixed for at least 48 h in 10% neutral buffered formalin prior to staining with 0.2% methylene blue (Georg T. Gurr, United Kingdom). The number, diameter, and localization of adenomas in the colon and small intestine were scored by transfusion in an inverse light microscope at a magnification of ×20. The diameters of the adenomas were scored with an eyepiece graticule. The tumor position along the intestines was recorded in cm from the ventricle. For each experimental group the incidence of tumors, defined as the number of mice with tumors/number of mice in the group, the mean number of tumors/mouse ± SD, and the mean tumor diameter (mm) in the group ± SD, was calculated for the small intestine and colon separately.

We have described recently a specific type of dysplastic ACF in the colons of Min+/ mice, denoted ACFSD (38, 39). In contrast to the classical ACF, the dysplastic ACF are not elevated above the surrounding mucosa and are visualized only after transillumination of the methylene blue-stained tissue. For each experimental group the incidence of dysplastic ACF, defined as the number of mice with dysplastic ACF/number of mice in the group, the mean number of dysplastic ACF/mouse ± SD, and the mean AC/ACF (crypt multiplicity) in the group ± SD, was calculated.

Induction and Analysis of PhIP-DNA Adducts. Min+/ mice of both sexes were given a single s.c. injection of 50 mg/kg PhIP on either day 12 (11–13) or 36 (35–36) after birth and were terminated 24 h after the injection. Untreated controls were terminated at the same age.

The colon and small intestine were removed separately and rinsed in ice-cold PBS. The small intestine was divided in three sections of equal length. The complete intestinal tissue, including the muscularis layer, was used for analysis because of the practical difficulties with separating the mucosa from the muscularis in these young intestines. The tissues were immediately frozen and stored at −70°C until analysis. DNA was isolated by homogenizing the entire section of tissue in nuclei lysis buffer [10 mM Tris-HCl, 400 mM NaCl, and 2 mM tetrasodium EDTA (pH 8.2)] and incubation with proteinase K as before (40). DNA was precipitated with salt and ethanol and then digested with RNase A and RNase T1. DNA was then extracted with phenol-chloroform-isooamy alcohol and with chloroform-isooamy alcohol solutions, after which it was quantitated spectrophotometrically using its absorbance at 260 nm.

PhIP-DNA adducts were quantitated using 32P-postlabeling under intensification (ATP-deficient) conditions (40, 41). In brief, the DNA samples were enzymatically digested with micrococcal nuclease (Sigma Chemical Co., St. Louis, MO) and spleen phosphodiesterase (Worthington, Lakewood, NJ). Using T4 polynucleotide kinase (United States Biochemical Corp., Cleveland, OH), the resulting 3′-nucleotide monophosphates were labeled with [γ-32P]ATP to form 3′,5′-bisphosphate nucleotides. The adducts were separated from normal nucleotides and resolved on polyethyleneimine cellulose thin-layer sheets using the following solvents: D1, 2.3 mM sodium phosphate (pH 5.8); D2, 2.81 mM lithium formate and 6.63 M urea (pH 3.5); D3, 0.8 M lithium chloride, 0.5 M Tris-HCl and 7.4 M urea (pH 8.0); and D4, 1.0 mM magnesium chloride. The labeled adducts were detected by autoradiography and quantitated using Cerenkov counting. The adduct levels were expressed as RAL values obtained under intensification conditions (<RAL>) and converted to actual RAL values using previously determined intensification factors (40).

Statistical Analysis. The data for tumor number and diameter and number of dysplastic ACF and AC/ACF were analyzed with one-way ANOVA followed by the Student-Newman-Keuls all pairwise multiple comparison procedure or with Kruskal-Wallis ANOVA on ranks for nonparametric data (SigmaStat software; Jandel Scientific, Germany). In addition, the Student’s t test or Mann-Whitney rank sum test for nonparametric data were sometimes used to compare two groups that did not reach statistical significance with one-way ANOVA. Fisher exact probability test (two-tailed probability) was used to evaluate incidence data. The DNA adduct data were analyzed by both two-way or three-way ANOVA and the Student’s t test/Mann-Whitney rank-sum test. A P of <0.05 was considered significant.

RESULTS

To study more closely the period of susceptibility to PhIP perinatally, we first defined the single dose of PhIP needed to induce intestinal tumors. Thereafter, the mice were exposed to PhIP in utero or at various time points after birth. We also examined whether one dose of PhIP via breast milk could induce tumors. The levels of PhIP-DNA adducts were quantitated and correlated to tumor levels for various time points of PhIP exposure and for different intestinal regions.

The very few statistically significant differences found between female and male Min+/ mice in the number or diameter of tumors in the small intestine or colon, or in the number of dysplastic ACF, did not show any consistent pattern, indicating no biological significance.
Therefore, in the following presentation of the results, data from female and male mice are pooled.

**Effects of a Single Dose of PhIP.** The effect of a single s.c. injection of PhIP was investigated by giving either 1, 10, or 50 mg/kg on day 3 after birth. The mice were terminated either 7 weeks after birth (10 and 50 mg/kg) or 11 weeks after birth (1, 10, and 50 mg/kg) as in our previous study (36). The 10 and 50 mg/kg PhIP doses induced a significant dose-dependent increase in the numbers of small intestinal tumors compared with the controls at weeks 7 and 11 ($P < 0.001$; Table 1). In the controls, the number of tumors increased significantly from week 7 to week 11 ($P < 0.001$; Table 1). This was also seen in the group given 50 mg/kg PhIP ($P = 0.002$). However, when the spontaneous tumors in the control mice were subtracted from the number of tumors in the PhIP-treated groups for each tumor size class at weeks 7 (Fig. 1A) and 11 (Fig. 1B), the PhIP-induced tumors were larger than the spontaneous tumors, indicating that the majority of the spontaneous tumors were initiated later than day 3 after birth; this assumes that spontaneous and PhIP-specific tumors grow at the same rate. Indeed, tumors induced by 10 mg/kg PhIP had apparently the same growth rate as the spontaneous tumors (Fig. 1). Tumors induced by 50 mg/kg PhIP, however, seemed to grow faster than both spontaneous tumors and tumors induced by 10 mg/kg PhIP, based on the differences in tumor size distributions observed between week 7 and week 11 (Fig. 1).

In the colon, PhIP-treatment resulted in a dose-dependent increase in both the incidence and number of tumors at both weeks 7 and 11 (Table 1). Only the high dose (50 mg/kg PhIP), however, increased the incidences ($P = 0.006$ and $P = 0.016$) and numbers ($P = 0.029$ and $P = 0.004$) of tumors significantly at weeks 7 and 11, respectively (Table 1). Except for an increase in the diameter of the colonic tumors from week 7 to week 11 in mice given 50 mg/kg PhIP ($P < 0.001$), there were no statistically significant differences in tumor size between the groups (data not shown).

The highest numbers of tumors were localized in the distal two-thirds of the small intestine, both in the PhIP-treated mice, independent of PhIP dose, and in the controls, in mice terminated at weeks 7 or 11 (data not shown). This is in agreement with our previous study using multiple injections of PhIP (36). No apparent effect of PhIP on localization of the few tumors present in the colon was observed (data not shown).

In addition to tumors, dysplastic ACF were scored in the colons. At week 11, both the incidence ($P = 0.028$) and number ($P = 0.032$) of dysplastic ACF were significantly lower after exposure to 50 mg/kg PhIP.

**Table 1. Effects of PhIP on the incidence and number of intestinal tumors and dysplastic ACF in Min/+ mice**

<table>
<thead>
<tr>
<th>Treatment (mg/kg PhIP)</th>
<th>Small intestinal tumors</th>
<th>Colonial tumors</th>
<th>Colonial dysplastic ACF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incidence $^a$</td>
<td>No. $^b$</td>
<td>Incidence $^a$</td>
</tr>
<tr>
<td>7 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>20 of 20</td>
<td>37.5 ± 21.3 $^e$</td>
<td>6 of 20</td>
</tr>
<tr>
<td>10</td>
<td>15 of 15</td>
<td>88.5 ± 16.3 $^{d,e}$</td>
<td>8 of 15</td>
</tr>
<tr>
<td>50</td>
<td>15 of 15</td>
<td>147.5 ± 22.9 $^{d,e}$</td>
<td>12 of 15$^d$</td>
</tr>
<tr>
<td>11 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>22 of 22</td>
<td>56.4 ± 15.0 $^f$</td>
<td>10 of 22</td>
</tr>
<tr>
<td>1</td>
<td>11 of 11</td>
<td>48.9 ± 12.1</td>
<td>3 of 11</td>
</tr>
<tr>
<td>10</td>
<td>12 of 12</td>
<td>92.4 ± 18.1 $^{d,e}$</td>
<td>6 of 12$^d$</td>
</tr>
<tr>
<td>50</td>
<td>16 of 16</td>
<td>168.4 ± 19.3 $^{d,e}$</td>
<td>14 of 16$^{d,e}$</td>
</tr>
<tr>
<td>Via breast milk</td>
<td>22 of 22</td>
<td>64.9 ± 15.3</td>
<td>7 of 22</td>
</tr>
</tbody>
</table>

$^a$ The number of mice with tumors or dysplastic ACF/mouse.

$^b$ The mean number of tumors or dysplastic ACF/mouse ± SD.

$^c$ Significantly different between 7 and 11 weeks ($P < 0.05$).

$^d$ Significantly different from the respective controls ($P < 0.05$).

$^e$ Significantly different between 10 and 50 mg/kg ($P < 0.05$).
All mice were terminated 11 weeks after birth. Data from females and males are pooled. 

**Susceptibility to PhIP Perinatally.** On the basis of the results obtained with a single s.c. PhIP injection, the 50 mg/kg dose was chosen in experiments to define more closely the susceptibility to PhIP perinatally. Min/+ pups were exposed in utero through a single s.c. injection of 50 mg/kg PhIP to the dams on day 3 before birth. Other groups of Min/+ pups were exposed postnatally by a single s.c. injection of 50 mg/kg PhIP on either day 3, 12, or 36 after birth. All mice were terminated at week 11.

The period of highest susceptibility to the effects of PhIP on tumor formation in the small intestine was apparently shortly after birth (Fig. 2A). About three (P < 0.001) or 2.6 (P < 0.001) times higher numbers of small intestinal tumors were induced when PhIP was injected on day 3 or 12 after birth, respectively, compared with the untreated controls. A much smaller increase in the number of small intestinal tumors was observed after exposure to PhIP in utero (P = 0.013) or on day 36 (P = 0.045) after birth. The exposure on day 3 after birth induced a significantly higher number of small intestinal tumors compared with those following exposure in utero or on day 12 or 36 after birth (P < 0.001; Fig. 2A). The tumor number was significantly higher after exposure to PhIP on day 12 compared with day 36 after birth (P < 0.001).

The diameter of the small intestinal tumors was significantly increased after exposure to PhIP in utero or on day 3 or 12 after birth compared with tumors in the control mice (P < 0.001; data not shown). The tumors induced by PhIP exposure on day 3 after birth were significantly larger than those induced by PhIP exposure in utero or on day 12 after birth (P < 0.001). After exposure to PhIP on day 36 after birth, however, diameters of small intestinal tumors were significantly lower than those of both spontaneous tumors and tumors induced by PhIP exposure either in utero or on day 3 or 12 after birth (P < 0.001; data not shown).

The effects of PhIP on tumor size are illustrated in the curves of the size distribution of PhIP-specific tumor populations (Fig. 3). As expected, the size of PhIP-induced tumors decreased with decreasing time for growth after PhIP injection except for the PhIP-induced tumors in mice exposed to 50 mg/kg PhIP on day 3 after birth, which was larger than those after exposure in utero, possibly indicating a more rapid growth of this tumor population. The size distribution of tumors from control mice indicated that spontaneous tumors are

Figure 2. Susceptibility to PhIP exposure perinatally. The number of tumors in the small intestine (A) and colon (B) of Min/+ mice (mean ± SD). The mice were exposed in utero through a single s.c. injection of 50 mg/kg PhIP to their dams on day 3 before birth or directly by a single s.c. injection of 50 mg/kg PhIP on either day 3, 12, or 36 after birth. All mice were terminated 11 weeks after birth. Data from females and males are pooled. * indicates the tumor number in the untreated control mice. ** significantly different from the controls (P < 0.05).

Figure 3. Size distributions of PhIP-induced tumors in the small intestine pooled from female and male Min/+ mice. The mice were exposed in utero through a single s.c. injection of 50 mg/kg PhIP to their dams on day 3 before birth or directly by a single s.c. injection of 50 mg/kg PhIP on either day 3, 12, or 36 after birth. All mice were terminated 11 weeks after birth. The PhIP-induced tumor populations were calculated by subtracting the spontaneous tumors formed in the controls from the tumors formed in the PhIP-treated mice for each tumor size class and are presented as tumor diameter (mm/mouse). The interval between tumor size classes is 0.25 mm.

The tumors induced by PhIP exposure on day 3 after birth were significantly larger than those induced by PhIP exposure in utero or on day 12 after birth (P < 0.001). After exposure to PhIP on day 36 after birth, however, diameters of small intestinal tumors were significantly lower than those of both spontaneous tumors and tumors induced by PhIP exposure either in utero or on day 3 or 12 after birth (P < 0.001; data not shown).

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In the colon, the incidence of tumors was significantly higher in mice exposed to PhIP in utero (16 of 18; \( P = 0.007 \)) and on day 3 (14 of 16; \( P = 0.016 \)) after birth compared with the controls (10 of 22). The numbers of tumors were 3.5 times (\( P < 0.001 \)) and 3.3 times (\( P = 0.004 \)) higher in these groups than in the controls, respectively (Fig. 2B). Exposure to PhIP on day 12 after birth was less efficient in inducing colonic tumors, as the number was not significantly different from that in the controls (Fig. 2B). When PhIP was injected on day 36 after birth, no effect on the number of colonic tumors was seen (Fig. 2B). The diameter of colonic tumors was not significantly affected by PhIP, irrespective of the time point of injection (data not shown). No influence of the time point of PhIP exposure was observed on the localization of tumors in either the small intestine or the colon (data not shown). Neither the number of dysplastic ACF nor the crypt multiplicity (AC/ACF) was significantly different from the controls, irrespective of the day of PhIP exposure pre- or postnatally (data not shown).

**PhIP-DNA Adducts.** To study whether the observed variation in susceptibility to PhIP-induced intestinal tumorigenesis, depending on age at exposure and intestinal region, was associated with the levels of DNA adducts formed, we used \(^{32}\)P-postlabeling to quantitate PhIP-DNA adducts in the intestines 24 h after exposure to PhIP. As described above, the highest number of small intestinal tumors was found after injection of 50 mg/kg PhIP on day 3 after birth. However, because of the difficulties with handling the intestines from mice this young, we compared the levels of PhIP-DNA adducts in mice exposed to PhIP on days 12 and 36 after birth. The number of small intestinal tumors but not colonic tumors was significantly higher after PhIP administration on day 12 compared with day 36 (\( P < 0.001 \)). We also compared the PhIP-DNA adduct levels between the colon and the proximal, middle, and distal small intestine to examine whether adduct levels correspond with tumor localization along the intestines.

As before (40, 41), four PhIP-DNA adducts were detected (data not shown). Adduct 1 is shown previously to be \( N \)-(deoxyguanosin-8-yl)-PhIP (42), which is the major PhIP-DNA adduct in all species examined (15). The identities of adducts 2 and 3 are unknown, but they may be dimers or higher mers of adduct 1 (15). Therefore, the results are presented as total levels of adducts 1–3 \((10^3 \times \text{RAL})\). A fourth adduct, designated adduct 5 to be consistent with numbering used previously (43), also with unknown identity, could not be detected as consistently as the other three and was present in much lower levels. Adduct 5, which is poorly separated from adduct 2, is undetectable when the assay is run under standard (ATP-deficient) conditions and hence, cannot be quantitated correctly. Assuming intensification to at least the same extent as adducts 1 and 2, adduct 5 would amount to <10% of the total in almost all of the samples. There were no statistically significant differences in the levels of either PhIP-DNA adducts 1–3 or 5 between female and male Min\(^+\) mice. Therefore, the results from female and male mice are pooled. No PhIP-DNA adducts were detected in unexposed control mice \((n = 4)\).

**Difference in PhIP-DNA Adduct Levels between Mice Injected on Day 12 or Day 36 Postnatally.** DNA adducts 1–3 were detected after injection of PhIP both on day 12 and on day 36 (Fig. 4), whereas adduct 5 was not detected in any samples after exposure on day 36 and only in some samples after exposure on day 12 (data not shown). There was a generally higher level of adducts 1–3 after exposure on day 12 compared with day 36 \((P < 0.0001)\). This was apparent in the proximal, middle, and distal parts of the small intestine \((P < 0.0001)\) but not in the colon.

**Variation in PhIP-DNA Adduct Levels along the Intestines.** The levels of adducts 1–3 varied significantly along the intestines after PhIP exposure on day 12 \((P < 0.0001)\); Fig. 4). The adduct level in the entire small intestine was significantly higher than that in the colon \((P < 0.0001)\). All three parts of the small intestine had higher levels of adducts compared with the colon: proximal \((P = 0.002)\), middle \((P = 0.001)\), and distal \((P < 0.0001)\). In addition, both the middle \((P = 0.027)\) and distal \((P = 0.006)\) part of the small intestine had higher adduct levels than the proximal part. On day 36, however, the levels of these adducts did not vary significantly between the intestinal regions (Fig. 4). Levels of adduct 5 changed in parallel with those of adducts 1–3 and because it could not be quantitated accurately (see above), its levels were not subjected to statistical analysis.

**Correlation between PhIP-DNA Adduct Levels and Tumor Numbers.** There was generally good correlation between levels of PhIP-DNA adducts and the number of PhIP-induced tumors (Fig. 5). The levels of PhIP-DNA adducts 1–3 were higher in the entire small intestine as well as in all three parts of the small intestine, but not in the colon, on day 12 compared with day 36 (Fig. 4). The total number of small intestinal tumors (Fig. 2A) as well as the numbers in the middle and distal parts of the small intestine, but not the number of colonic tumors (Fig. 2B), were significantly higher after exposure on day 12 compared with day 36.

Both the levels of PhIP-DNA adducts and the number of PhIP-induced tumors were much higher in the small intestine than in the colon (Fig. 5). In the small intestine, the highest levels of PhIP-DNA adducts were found in the middle and distal parts, corresponding to where the majority of PhIP-induced tumors was localized, i.e.,, in the distal two-thirds of the small intestine (Fig. 5).

**DISCUSSION**

The major findings in this study are: (a) that by using a single s.c. injection of PhIP to induce intestinal tumors, the most susceptible period to PhIP was found to be the perinatal period; (b) in contrast to the small intestine, which was most susceptible to PhIP shortly after birth, the colon was susceptible to tumor induction by PhIP also before birth; and (c) the levels of PhIP-DNA adducts largely correlate with the tumor levels both for the various time points of PhIP exposure and for the different intestinal regions.

**Effects of a Single Dose of PhIP.** Our study shows clearly that even a single s.c. injection of 50 mg/kg PhIP, or to a lesser degree, 10
mg/kg PhIP, is enough to strongly affect tumor induction in the colon and small intestine of both sexes of neonatal Min+/+ mice. Others have shown that a single i.p. injection of 50 mg/kg N-ethyl-N-nitrosourea increased intestinal tumorigenesis in neonatal Min+/+ mice (44).

Previously, we studied the induction of intestinal tumors in neonatal Min+/+ mice given multiple s.c. injections of PhIP (three times/week totaling eight injections of 50 mg/kg; Ref. 36). However, a single s.c. injection of 50 mg/kg PhIP used in this study only induced a slightly lower (1.4-fold) mean number of small intestinal tumors (168.4) than eight injections (228.0), although these injections were given within the period of high susceptibility. The mean tumor diameter was slightly lower after eight injections (0.96 mm) compared with one injection (1.18 mm), probably reflecting the continuous induction of slightly lower (1.4-fold) mean number of small intestinal tumors (168.4) than eight injections (228.0), although these injections were given within the period of high susceptibility. The mean tumor diameter was slightly lower after eight injections (0.96 mm) compared with one injection (1.18 mm), probably reflecting the continuous induction of small (small intestinal and colonic) tumors in Min+/+ mice exposed on days 5–14 than on days 15–35 after birth (44).

By comparing the size distributions of PhIP-specific tumors and spontaneous tumors found in control mice (Fig. 3), it could be inferred that spontaneous tumor formation in the small intestine probably starts in utero and that the majority of tumors is formed during the first few weeks after birth. It seems that the period of susceptibility to PhIP coincides with the period of spontaneous tumor formation. When comparing the size distribution of PhIP-specific tumors induced by 10 and 50 mg/kg PhIP at weeks 7 and 11 (Fig. 1), it seems likely that the higher dose of PhIP also increases tumor growth. It could be speculated that this is due to a general effect of PhIP on cell proliferation in the intestinal mucosa as has been shown in the rat colon (47).

It has been known for a long time that neonatal animals or humans have an increased susceptibility to carcinogens compared with adults, and a number of factors have been suggested influencing susceptibility at different stages (48–51). These factors include a different ratio between body weight and target organ weight affecting the number of target cells at risk, differences in cell proliferation, apoptosis, or differentiation, or in the ability to absorb, metabolize (activate or detoxify), or excrete xenobiotic compounds, as well as in the repair of genotoxic insults. Interestingly, PhIP has been shown to be extremely toxic to XPA+/−/− knockout mice, which are defective in nucleotide excision repair (52). Also, in theory, antigenic neoplastic cells may be protected by immune tolerance as a consequence of emerging prior to functional maturation of the immune system, or susceptibility may be affected by the immature endocrine system.

Differences between neonatal and adult Min+/+ mice in the activity of both Phase I and Phase II enzymes involved in the activation of PhIP to an ultimate carcinogen are likely to contribute to the observed differential susceptibility to PhIP. It has for example been shown that the N-hydroxylation of PhIP, being the first step in its metabolic activation, increases about 3-fold from day 8 to day 15 after birth and thereafter increases only slightly to day 30 in C57BL/6 mice (53). Although the formation of DNA adducts is necessary for induction of cancers by genotoxic carcinogens, there is often no direct correlation between adduct levels and tumor induction or between the frequency of mutations formed from the DNA adducts and cancer (54, 55). However, in this study the formation of PhIP-DNA adducts could at least partly explain the differential susceptibility to tumor induction by PhIP, because we found an apparent association between the PhIP-DNA adduct levels and numbers of tumors when we compared PhIP-DNA adduct levels between Min+/+ mice exposed to PhIP at different time points after birth showing different susceptibility to of the effects observed on small intestinal tumors, the PhIP dose transferred in breast milk in this study apparently was <10 mg/kg and probably closer to 1 than to 10 mg/kg. This is in the same order of magnitude as previously measured values for transfer of PhIP in breast milk (46).
intestinal tumor induction, *i.e.*, day 12 and 36 (Fig. 5). There was a generally higher PhIP-DNA adduct level after exposure on day 12 compared with that on day 36, which was apparent in the proximal, middle, and distal parts of the small intestine but not in the colon. The number of small intestinal tumors but not colonic tumors was significantly higher after exposure to PhIP on day 12 compared with that on day 36 after birth. In addition, no difference in the number of dysplastic ACF was found between the colons from mice exposed to PhIP on day 12 and those exposed on day 36, which is also in accordance with the similar adduct levels observed in the colon at these time points.

Lower levels of DNA adducts were found in tumors than in the surrounding normal mucosa in the small intestines of *Min/+* mice exposed to benzo[a]pyrene (56). Therefore, if more tumors were present in the intestines on day 36 than on day 12 in our study, this could be the reason for the lower PhIP-DNA adduct levels found on day 36 compared with day 12. However, this is not likely because the total area of spontaneous tumors, which are the only tumors present 24 h after the PhIP injection when the mice were terminated, is very small compared with the area of the surrounding normal mucosa. In addition, if this was the case, there should have been a negative correlation between the number of tumors and DNA adduct levels between the various parts of the small intestine; in fact, the opposite was found.

The critical period of susceptibility observed in rodents cannot be transferred directly to humans because of the obvious differences across the species. For instance, some developmental events occur postnatally in rodents but prenatally in humans, and the “childhood” period is very short in rodents compared with that in humans. However, we have shown in this study that *Min/+* mice, with only one functional allele of the *Apc* gene, are highly susceptible to the effect of PhIP on tumor induction when exposed at an early age. The *Min/+* mouse is an excellent model for the dominantly inherited autosomal disorder FAP as well as for sporadic colorectal cancer, both conditions being caused by various mutations in the human APC gene. These results might be of particular relevance to children having inherited the human FAP syndrome carrying a mutated APC allele, especially because PhIP is demonstrated to be transferred to the offspring both *in utero* and through the breast milk (46). Although it is not possible to define exact periods of susceptibility for humans based on these results in mice, this study emphasizes the importance of exposure to carcinogens early in life.

**Regional Variation in Susceptibility to PhIP-induced Tumorigenesis along the Intestines.** We also compared the regional variation along the intestines in levels of PhIP-DNA adducts and number of tumors and found a good correlation. The highest levels of PhIP-DNA adducts were in the middle and distal parts of the small intestine, where tumor numbers were also the highest (Fig. 5). Much lower PhIP-DNA adduct levels and numbers of tumors were found in the colon. This is in agreement with our previous study in Syrian hamsters, where we found a lower level of N-(deoxyguanosin-8-yl)-PhIP in the distal part of the colon compared with that in the small intestine (57). However, no PhIP-related induction of tumors or ACF was found in that animal model, neither in rapid nor slow acetylator hamsters, and hence, a correlation between adduct levels and tumor induction could not be examined.

Variations in genotoxicity or mutation levels between intestinal regions apparently do not always correlate directly with tumor induction. After 2–10 i.p. or p.o. exposures to PhIP, no difference in *Dib-1* locus mutations was found between the proximal and distal small intestine of adult mice with a C57Bl/6d background (58). After an i.p. injection of 40 mg/kg PhIP in adult CD-1 mice, genotoxicity was detected with alkaline single cell gel electrophoresis assay in the colon but not in the duodenal, jejunal, or ileal part of the small intestine (59).

In theory, there are many possible reasons for the observed regional variation intrinsic to the intestinal mucosa, including variations in activating or detoxification enzymes (60, 61), in the expression of various modifier genes (62), or in processes such as proliferation, differentiation, and apoptosis (63, 64). In human mucosa, a much lower total level of unidentified DNA adducts was found in the small intestine compared with the colon (65), which is in agreement with the known prevalence of tumors. However, humans are exposed to carcinogens such as heterocyclic amines through their food intake, and the intestinal tumorigenesis is therefore also affected by the composition of the diet, the secretion of bile, and the intestinal bacterial flora, factors which all may affect the intestinal regions differently. In addition, the transit time is shorter and the intestinal content more diluted in the small intestine than in the colon, resulting in lower exposure to food carcinogens in the small intestine than in the colon.

In summary, we have shown that PhIP given as a single injection, or to a lesser extent, even as a single dose via breast milk, is a potent inducer of intestinal tumorigenesis in the *Min/+* mice, that the most susceptible age for PhIP exposure is the perinatal period, and that the formation of PhIP-DNA adducts could at least partly explain the differential susceptibility to tumor induction by PhIP that was observed between the various time points of PhIP exposure and the different intestinal regions.

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**REFERENCES**


Intestinal Tumorigenesis in Multiple Intestinal Neoplasia Mice Induced by the Food Mutagen 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine: Perinatal Susceptibility, Regional Variation, and Correlation with DNA Adducts

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