

Induction of Intestinal Tumors and Lymphomas in C57BL/6N Mice by a Food-borne Carcinogen, 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most abundant heterocyclic amine contained in cooked meat and fish. Although PhIP has been demonstrated to induce various types of tumors in rats, lymphomas predominated in mice using the CDF1 strain. To investigate the carcinogenic activity of PhIP on other organs in mice with a different genetic background, PhIP was administered to C57BL/6N mice. After a 40-week administration of 300 ppm of PhIP in a high-fat diet followed by continuous feeding with a high fat diet, C57BL/6N mice developed adenomas and adenocarcinomas in the small intestine, the incidences being 52% in males and 68% in females at weeks 95 and 70, respectively. Lymphomas of B-cell origin also developed in both sexes as frequently as in the CDF1 strain, incidences being 48% in males and 32% in females. Although the incidence in PhIP-treated female mice did not differ from that in the control mice, lymphomas developed significantly earlier in the PhIP-treated mice. The present study demonstrated that the intestinal tract is another potential target of PhIP-induced carcinogenesis in mice, and that the carcinogenic activity of PhIP could be affected by the genetic background of the animals.

Key words: PhIP — Strain difference — Intestinal tumor — Lymphoma — Mice

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is the most abundant mutagenic and carcinogenic heterocyclic amine contained in cooked meat and fish.¹⁾ PhIP has been shown to induce colon cancers,^{2,3)} prostate cancers⁴⁾ and lymphomas⁵⁾ in F344 male rats. Cecum and colon cancers are induced in Nagase analbuminemic male rats,⁶⁾ and mammary cancers in F344³⁾ and Sprague-Dawley female rats.⁷⁾ Despite this wide range of organs as carcinogenic targets of PhIP in rats, only lymphomas have been reported to be induced in mice after a long-term exposure using the CDF1 strain.⁸⁾ Recently, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), which is another type of heterocyclic amine and is widely used for carcinogenesis studies in rodents, was demonstrated to induce colon, cecum and liver cancers in C57BL/6N mice, and the target organs of MeIQ in C57BL/6N were different from those in CDF1.⁹⁾ This indicates that target organs of heterocyclic amines could vary among different mouse strains, and substantial contributions by the genetic backgrounds of mice need to be taken into consideration. In the present study, the carcinogenic activity of PhIP in mice was investigated using the C57BL/6N strain. C57BL/6N mice have been widely utilized for a variety of animal

experiments, including gene-disruption studies, and are highly susceptible to the induction of colon cancers by a synthetic alkylating agent, 1,2-dimethylhydrazine (DMH).^{10,11)} A 40-week feeding of 300 ppm PhIP in a high-fat (HF) diet, followed by a continuous feeding of the HF diet alone, was conducted and the carcinogenic activity of PhIP in various organs was analyzed.

MATERIALS AND METHODS

Experimental protocol C57BL/6N mice of both sexes were purchased from CLEA Japan (Tokyo) at the age of 6 weeks. PhIP was purchased from the Nard Institute (Osaka) and added to the diet. After a one-week acclimatization to the housing conditions and an AIN-93G diet, 26 or 27 mice of both sexes were fed a HF diet, a modified AIN-93G diet (Dyets, Bethlehem, PA) supplemented with 23% hydrogenated vegetable oil (PRIMEX; Dyets), containing 300 ppm of PhIP. After a 40-week administration of PhIP, mice were continuously fed the HF diet until the termination of experiment. Male and female mice fed only the HF diet without PhIP were used as controls.

Histological examination The mice were sacrificed and autopsied when they became moribund or at the termination of experiments. All organs were carefully examined macroscopically for the presence of tumors and then fixed in neutralized 10% formalin at 4°C, and embedded in paraffin blocks. Paraffin sections were cut into 3.5 μm thick-

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ness, then stained with hematoxylin-eosin (H&E), and histopathological analyses were performed under light microscopic observation.

Immunostaining of lymphocyte cell-surface antigens

Immunohistochemical staining of T- and B-cell surface antigens was carried out as follows. Paraffin sections of thymus, spleen and lymph nodes were prepared as described above. Monoclonal antibodies of anti-CD3 (CD3-12, Novocastra Lab., Newcastle upon Tyne, UK) and biotinylated anti-CD45R/B220 (RA3-6B2, PharMingen, San Diego, CA) were used to detect T- and B-cell surface antigens, respectively. Sections were pretreated in an autoclave for 10 min at 120°C in 1 mM EDTA (pH 8.0) for CD3, and 10 mM citrate buffer (pH 6.0) for CD45R/B220, and the ABC reagent (VECTASTAIN Elite ABC kit, Vector Lab., Burlingame, CA) was used to visualize the respective cell surface antigens.

Statistical analysis Survival rates were calculated by the Kaplan-Meier method. The differences in survival rates and cumulative incidences of tumors were analyzed by a log-rank test, and tumor incidences by χ^2 test. All statistical analysis was performed using the SPSS software for Macintosh (SPSS Japan, Inc., Tokyo), and the differences were considered to be significant when the *P* values were less than 0.05.

RESULTS

Effects of PhIP on survival Administration of PhIP caused the body weights of surviving male and female mice to gradually decrease to approximately 80% of the control by experimental week 40, and they stayed within the range of 80–90% of the control until the termination of experiments (data not shown). The first male mouse died of cancer at week 28, of adenocarcinoma in the small intestine, and the first female died of lymphoma at week 33. All the male and female mice that died after these respective dates were counted as effective for the carcinogenesis study. Some of the mice developed tumors in two

or more organs. The experiments were terminated at 95 and 70 weeks for male and female mice, respectively (Fig. 1). Survival rates of PhIP-treated mice were lower than those of untreated mice of both males and females ($P < 0.05$).

Carcinogenicity in the gastrointestinal tract In the gastrointestinal tract, small intestinal tumors were observed in 52% of male and 68% of female mice by the termination of experiments, and tumor incidences in both sexes were significantly higher than those of untreated mice (Table I). The majority of tumors in PhIP-treated groups were adenocarcinomas (Fig. 2A), accounting for 81% (17 of 21) in males and 86% (24 of 28) in females, with the remainder

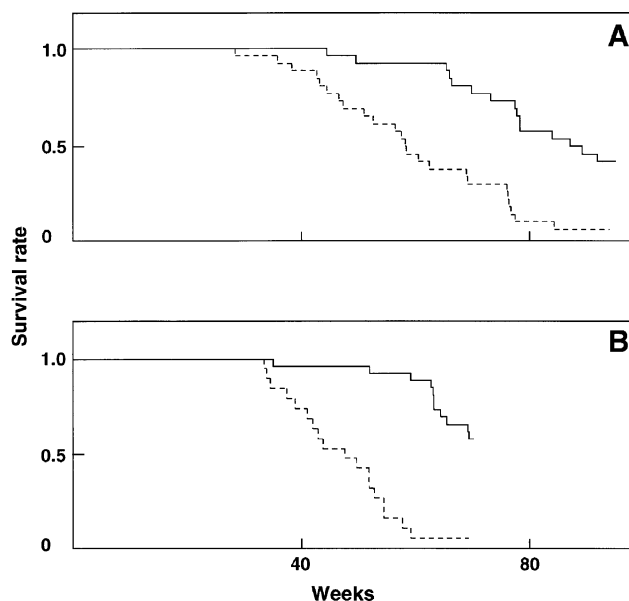


Fig. 1. Survival curve of C57BL/6N male (A) and female (B) mice. Solid lines (—) represent the survival of mice in PhIP-untreated control groups. Dashed lines (---) indicate the survival in PhIP-treated groups.

Table I. Tumor Development in C57BL/6N Mice Fed 300 ppm PhIP in Diet

Mice strain	Group	Sex	Treatment	Effective No.	No. of mice with tumors (%)				
					Small intestine	Cecum	Lymph node	Liver ^{a)}	Lung
C57BL/6N	I	M	None	25	3 (12)	0 (0)	3 (12)	20 (80)	1 (4)
			PhIP	25	13 (52)*	3 (12)	12 (48)*	10 (40)	0 (0)
	IV	F	None	25	1 (4)	0 (0)	8 (32)	1 (4)	3 (12)
			PhIP	19	13 (68)**	0 (0)	6 (32)	1 (5)	0 (0)

a) One adenoma and 2 hepatoblastomas in group I, and 1 adenoma and 1 hepatoblastoma in group II are also included.

* $P < 0.01$ in comparison with group I by χ^2 test.

** $P < 0.01$ in comparison with group III by χ^2 test.

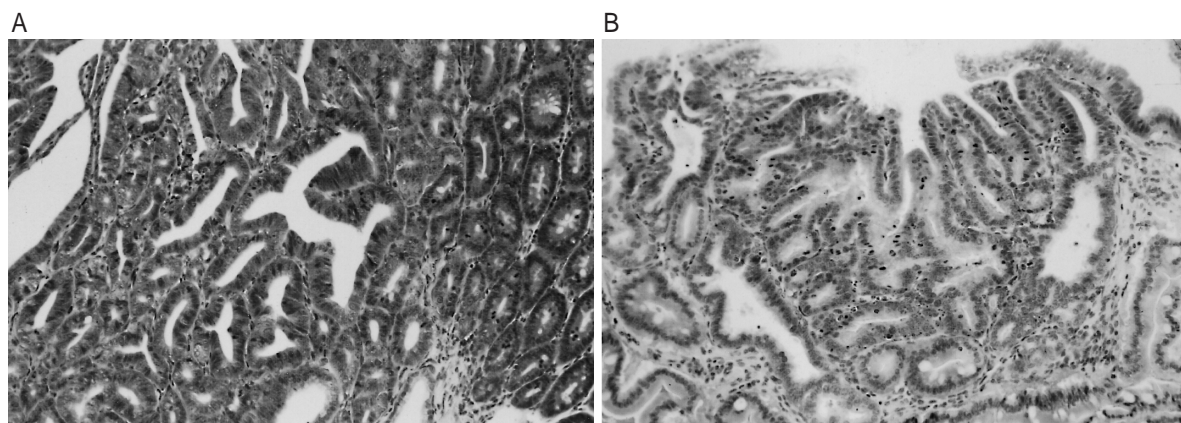


Fig. 2. Microscopic findings of adenocarcinoma (A) and adenoma (B) in small intestines of C57BL/6N female mice treated with PhIP. A: The tumor demonstrates severe structural and cellular atypia, and invades into the surrounding normal crypts. B: The tumor is localized in the mucosal area and is composed of cells with mild cellular atypia. H&E staining, $\times 50$.

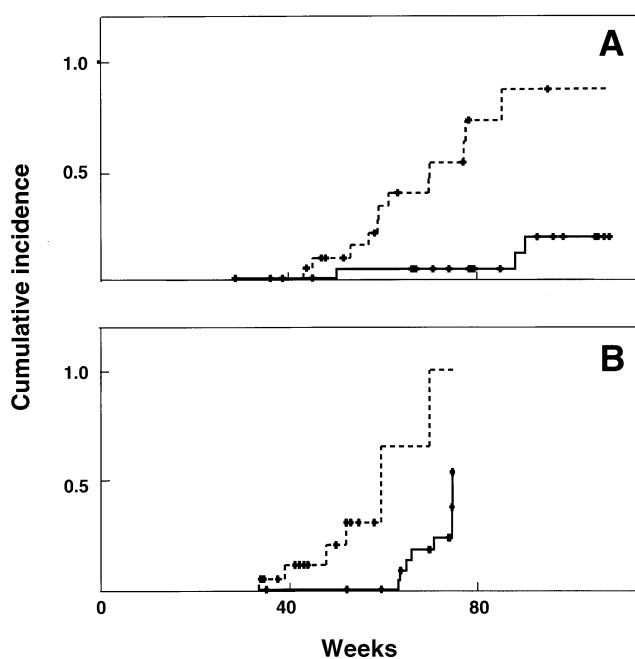


Fig. 3. Cumulative incidences of lymphoma of C57BL/6N male (A) and female (B) mice. Solid (—) and dashed lines (---) represent cumulative incidences of lymphomas in the PhIP-untreated and PhIP-treated groups, respectively. Crosses (+) indicate the censored cases, which died of causes other than lymphomas.

being adenomas (Fig. 2B). Four tumors that developed in the control groups, 3 in males and 1 in a female, were also diagnosed as adenocarcinomas. No difference in histopathological appearance or grade of tumors was observed

between PhIP-treated and control groups. Tumor sites were distributed along the entire small intestine, but mostly in the middle and proximal parts (data not shown). Three male mice in the PhIP-treated group developed adenocarcinomas in the cecum. No tumors were detected in the esophagus, stomach or colon.

Carcinogenicity in lymphoid tissues Administration of PhIP also increased the incidence of lymphoma in male mice (Table I, $P < 0.01$). In female mice, although no increase was observed in its incidence, lymphomas developed much earlier in PhIP-treated mice than in the control mice, as is depicted in Fig. 3 ($P < 0.05$). Lymphomas developed mainly in the mesenchymal lymph nodes (Fig. 4A), and occasionally in the liver, spleen and lungs (Fig. 4B). Five of 12 lymphomas that developed in PhIP-treated male mice were subjected to immunohistochemical analysis. All of them were mainly composed of CD45R/B220 positive cells with a few CD3-positive cells (Fig. 5), and were diagnosed as lymphomas of B-cell origin. Lymphomas that developed in PhIP-treated female mice and those in control mice were also of B-cell origin (data not shown), and no differences were observed histologically between PhIP-treated and control groups.

Carcinogenicity in other organs Liver tumors, being mostly hepatocellular carcinomas and occasionally adenomas or hepatoblastomas, developed at a high frequency especially in male mice, the incidences being 80% in the control mice and 40% in the PhIP-treated mice. In the lung, 4 tumors (1 adenocarcinoma in a male and 3 adenomas in females) developed spontaneously in the control groups, but no tumor was induced in the PhIP-treated groups. None of the mice of either sex developed tumors in mammary glands or prostate gland by macroscopic observation.

DISCUSSION

As described earlier, when PhIP was administered in the diet, predominant induction of lymphomas has so far been reported in mice,⁸⁾ although PhIP has been found to induce mutations predominantly in the intestines using “Muta,”¹²⁾ *lacI*,¹³⁾ *Dlb-I^{a/b}*¹⁴⁾ and *gptΔ*¹⁵⁾ transgenic mice. The present study demonstrated that C57BL/6N mice were susceptible to the induction of intestinal tumors, mainly in the small intestine, and of lymphomas after a 40-week continuous feeding of PhIP on a HF diet. Our results indicate an apparently different spectrum of target organs from that of PhIP in CDF1 mice.⁸⁾ These differences are considered to be caused by different genetic effects between

C57BL/6N and CDF1 mice. Modifying effects by genetic background on the development of intestinal tumors have also been reported in DMH-induced colon carcinogenesis in mice.^{10,11)} Klein *et al.*¹⁶⁾ recently reported that DNA repair-deficient *XPA*^{-/-} mice, generated on the C57BL/6 genetic background, were extremely susceptible to intestinal toxicity of PhIP and to the development of tumors in the small intestine. They also observed the development of one small intestinal adenoma in one of seven wild-type C57BL/6 female mice after a 3-month exposure to 25 ppm of PhIP in diet with an additional observation period of 9 months.

It is interesting to note that the majority of intestinal tumors arose in the small intestine, not in the colon, which

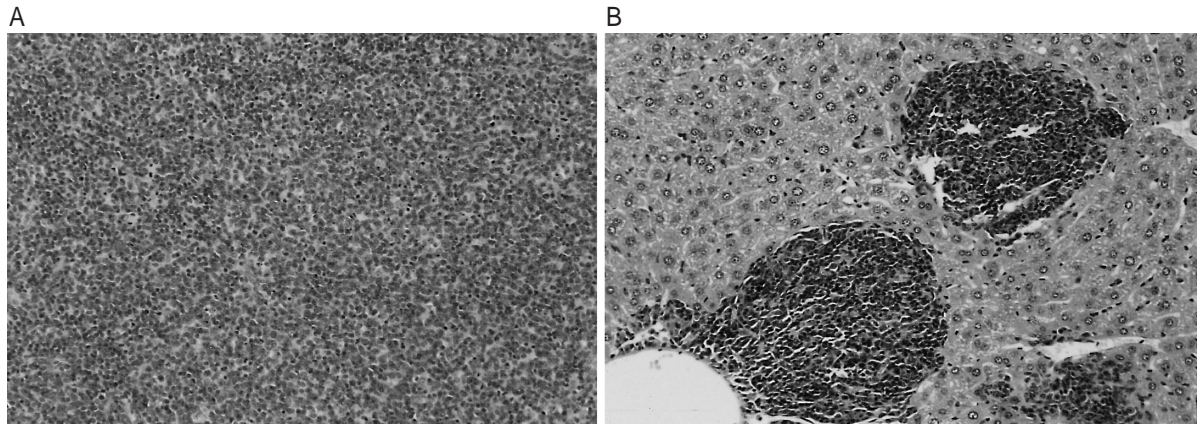


Fig. 4. Microscopic findings of lymphomas in a mesenchymal lymph node (A) and invasion of lymphoma in the liver (B) of a C57BL/6N male mouse treated with PhIP. H&E staining, $\times 50$.

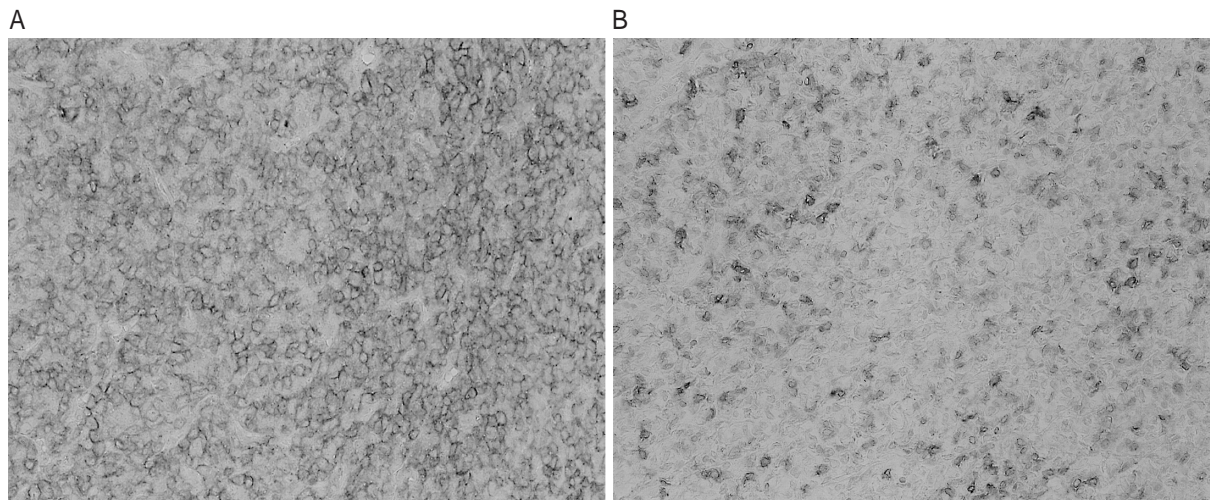


Fig. 5. Immunohistochemical staining of a lymphoma induced in a C57BL/6N male mouse, $\times 80$. A, anti-CD45R/B220 antibody (B-cell marker); B, anti-CD3 antibody (T-cell marker).

is one of the main target organs of PhIP in rats.^{2,3)} Since the level of PhIP-DNA adducts in the small intestine were demonstrated to be almost equivalent to that in the colon,¹⁷⁾ some other unknown modifying factors may exist to generate the differential susceptibility between the small intestine and colon in mice.

C57BL/6N mice were also susceptible to the induction of lymphomas of B-cell origin. In female mice, although the incidence of lymphomas did not differ statistically from the control group, the average experimental duration when lymphomas were detected was significantly different from that of the control mice. It is indeed important to take into consideration the effect of death due to other tumors on survival time, when analyzing carcinogenic activities and target organ spectra of carcinogenic compounds. A decrease in liver tumor incidence in male mice is also due to the reduction of the survival time in PhIP-treated mice.

The data presented in this report demonstrate the susceptibility of C57BL/6N mice to small intestinal tumors and B-cell lymphomas, and genetic background is consid-

ered to be one of the key determinants for the susceptibility of mice to an environmental carcinogen, PhIP. A body of carcinogenesis studies of various compounds has been assessed, though only using a relatively limited number of mice strains. The results of the present study suggest to us that carcinogenesis studies should be conducted using various species and strains of animals in order to investigate the potential carcinogenic activity of PhIP and in its relevance to human carcinogenesis.

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