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Dose-dependent Induction of Mammary Carcinomas in Female Sprague-Dawley Rats with 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

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The dose-dependence of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) induction of mammary carcinomas was investigated in female Sprague-Dawley (SD) rats given PhIP in the diet for 48 weeks at concentrations of 0, 25, 100 and 200 ppm in experiment 1, and 0, 12.5, 50 in experiment 2. Yields of ductular lesions, including intraductal papillomas and carcinomas, as well as papillo-tubular and solid-tubular carcinomas, showed dependence on the dose, with the respective total incidences being 0, 4.8, 25, 72.2 and 0, 10 and 35%. There was thus no apparent carcinogen exposure threshold. The present results confirmed the carcinogenicity demonstrated in a previous study using F344 rats and revealed the SD rat strain to be more susceptible.

Key words: PhIP — Mammary carcinoma — SD rat — Dose response

In recent years, a number of heterocyclic amines present in charred parts of broiled fish and meat have been demonstrated to be highly mutagenic in *Salmonella typhimurium* and also to exert tumorigenic potential in a variety of organs in either mice or rats; they are therefore considered as strong candidates for human carcinogens.[1-3] One in particular, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), was identified as the most abundant mutagenic heterocyclic amine produced in cooked meat and fish.[3,4] PhIP has also been found in beer and wine.[5] It has been reported that no PhIP was detected in smoke,[6] but PhIP was reported to be detected in cigarette smoke condensate.[7] Although its mutagenic potency in the *Salmonella typhimurium* assay is relatively low, PhIP was demonstrated to be more mutagenic than other heterocyclic amines in cultured mammalian cells,[8,9] and it induces DNA adducts, as demonstrated by ^{32}P-postlabeling analysis, in a variety of organs, including the heart, lung, pancreas and colon.[10,11] It has been shown to induce lymphomas in CDF_1 mice[12] and colon and mammary carcinomas in rats.[13,14] In our previous study, PhIP was given to male and female F344 rats at concentrations of 0, 25 or 100 ppm in the diet for two years, and a dose-dependency of PhIP carcinogenicity was observed in the colon of males and in the mammary glands of females.[15,16] An increased risk of mammary carcinoma development was also observed following transplacental and trans-breast milk exposure in Sprague-Dawley (SD) rats.[17]

In the present study, the dose-dependence of PhIP mammary carcinogenicity was further investigated in female SD rats, a strain which is generally more sensitive than F344 rats in terms of mammary tumorigenesis, and at the same time, cell proliferation of the mammary glands was examined during the course of carcinogenesis.

MATERIALS AND METHODS

PhIP (PhIP hydrochloride), produced by the Nard Institute, Osaka, was kindly provided by the National Cancer Center, Tokyo. Its purity was more than 99.9% by HPLC analysis, UV spectroscopy and elemental analysis. It has been confirmed by HPLC analysis that more than 90% of PhIP in diet stored at room temperature for one year remains unchanged.

Female Crj:CD (SD) rats (Charles River Japan Inc., Atsugi) at 5 weeks of age were randomly divided into groups and housed 5 per cage with wood-chip bedding in an air-conditioned animal room at 24±2°C and 55±5% humidity. When the rats were 6 weeks old, the experiment was started (week 0) and PhIP was mixed in the diet (Oriental MF powdered diet, Oriental Yeast Co., Tokyo) at the concentrations given below. In experiment 1, 4 groups of 30 rats each were given PhIP at concentrations of 0 (control), 25, 100 and 200 ppm, respectively. In experiment 2, started about 7 months later than experiment 1, 3 groups of 30 rats each were given PhIP at concentrations of 0 (control), 12.5 and 50 ppm. Food and water were available *ad libitum* throughout the experiment, and body weight and food consumption data were recorded every 2 weeks until the end of the experiment. Palpable tumors first appeared at week 22 and such

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lesions were counted at two-week intervals from this time point. In both experiments 1 and 2, at weeks 12, 24 and 48, BrdU (Sigma Chemical Co., St. Louis, MO) was administered to 5 rats of each group by single i.p. injection 1 h before the animals were killed.

In all other cases, surviving rats were killed at the end of week 48 under light ether anesthesia. After careful gross examination, mammary tissue with skin and any subcutaneous nodules were dissected along with the major internal organs, fixed in buffered formalin and routinely processed for histological examination of hematoxylin and eosin-stained sections. The intestines were inflated with fixative before being opened longitudinally and processed as rolls after the entire mucosal surface had been grossly examined. The livers, kidneys and spleens were weighed before processing.

Statistical analysis was performed using Fisher's exact probability test for incidence data and Student's t test or Welch's t test in combination with the F test for variability of means between any two groups.

RESULTS

The growth curves of rats in both experiments are shown in Fig. 1. Reduced body weight gain was observed in a PhIP-dose-dependent manner throughout the experiment. The average food intake, estimated total PhIP intake, body weight, relative liver, kidney and spleen weights at week 48 are listed in Table I. The total PhIP intake was in good relation with the amount of PhIP mixed in the diet except in the 200 ppm group case, where food intake could not be accurately measured because the rats always raked the powder diet out of the diet containers. The final body weights were significantly reduced by the PhIP treatment, and the relative weights of the liver, kidney and spleen, with respect to the body weight, had a tendency to increase with PhIP treatment. Although the experimental conditions for both experiments 1 and 2 were exactly the same, except for the 7 months' interval between them, slight differences in body weight and average food consumption values were observed in both treatment and control groups, probably due to differences in batches of rats or unidentified seasonal factors.

PhIP dose- and time-dependent induction of mammary tumors was clear, as demonstrated in Figs. 2 and 3, which respectively show cumulative incidence and multiplicity data for palpable mammary tumors. Data for mammary tumors observed histopathologically at week 48 are summarized in Table II. Most were confirmed to be papillo-tubular carcinomas. The induction of adeno-

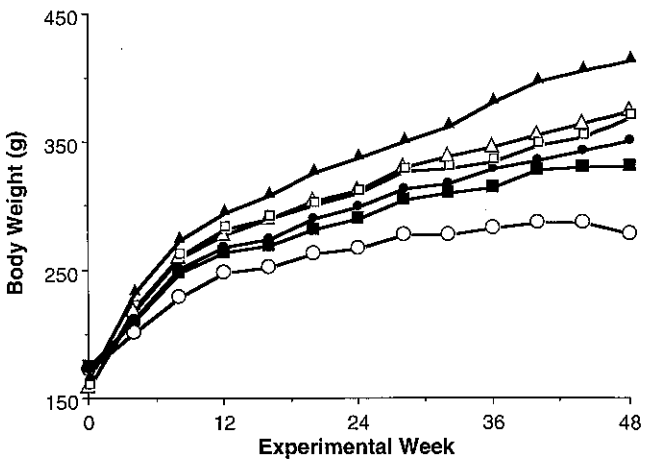

Fig. 1. Body weight curves of SD female rats treated with PhIP. ○ 200 ppm, ■ 100 ppm, △ 50 ppm, ● 25 ppm, □ 12.5 ppm, ▲ control.

Table I. Final Body, Relative Organ Weights, Average Food Intake and Total Intake of PhIP by Week 48

PhIP dose (ppm)	No. of rats	Body weight (g)	Weight (% body weight)			Average food intake (g/rat/day)	Total PhIP intake (mg/rat)
			Liver (%)	Kidney (%)	Spleen (%)		
Experiment 1							
0	20	421±81	2.65±0.23	0.25±0.03	0.14±0.02	14.00	0
25	21	353±46**	2.86±0.30*	0.29±0.04**	0.15±0.02	14.35	120.6
100	20	333±37**	3.06±0.24***	0.30±0.04***	0.18±0.08*	14.16	475.9
200	15	279±36***	3.16±0.38***	0.34±0.03***	0.20±0.07**	17.31	1163.3
Experiment 2							
0	19	408±50	2.70±0.23	0.26±0.03	0.15±0.20	14.94	0
12.5	20	370±47*	3.01±0.31**	0.29±0.04*	0.16±0.02	15.87	66.6
50	20	376±60	2.98±0.23***	0.29±0.03**	0.16±0.02	17.42	292.6

*, **, ***; Significantly different from the control values at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

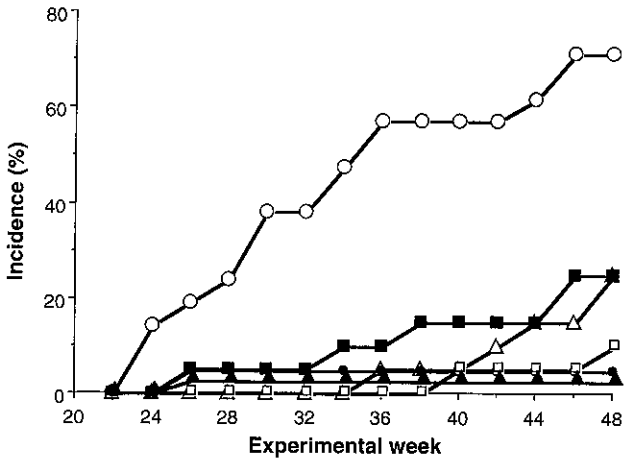

Fig. 2. Cumulative incidence data for palpable mammary tumors of SD female rats treated with PhIP. ○ 200 ppm, ■ 100 ppm, △ 50 ppm, ● 25 ppm, □ 12.5 ppm, ▲ control.

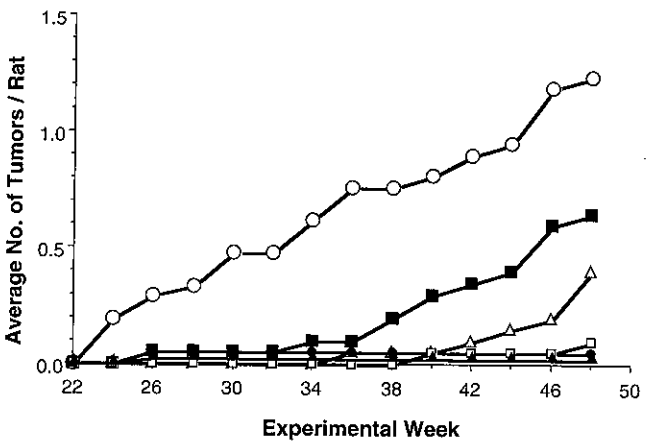

Fig. 3. Multiplicity data for palpable mammary tumors of SD female rats treated with PhIP. ○ 200 ppm, ■ 100 ppm, △ 50 ppm, ● 25 ppm, □ 12.5 ppm, ▲ control.

carcinomas was PhIP-dose-dependent and was observed in rats given as little as 12.5 ppm PhIP. The incidences of adenocarcinomas in rats given more than 50 ppm PhIP were statistically significant. Values in experiment 2 were slightly higher than in experiment 1. There was no clear dose-dependent tendency regarding the distribution of tumors (Table III). Metastases were not found.

No colon tumors were observed in PhIP-treated rats and no remarkable changes were noted in other organs, including the liver, kidney, pancreas, lung and stomach.

DISCUSSION

In an earlier study of PhIP, dose-dependent colon and mammary carcinogenicity was found in F344 rats administered this heterocyclic amine in the diet at doses of 400 ppm for 52 weeks[13] and 100 ppm and 25 ppm for 104 weeks.[17] In the latter case, the adenocarcinoma incidences were 47 and 7%, respectively, with one case of metastasis to the lung being observed. In the present experiment, the adenocarcinoma incidence was 72% in the 200 ppm group treated for 48 weeks. Thus, the present experiments confirmed the carcinogenicity of PhIP for the mammary gland observed earlier in F344 rats[15] and showed this tissue in the SD strain to be specifically more susceptible with a clear dose dependence. In the present studies, BrdU labeling indices of non-neoplastic mammary glands at weeks 12, 24 and 48 were 0.10 to 0.26 in the control group and 0.02 to 0.29 in the PhIP-treated group in experiment 1, and 0.39 to 0.98 in the control group and 0.06 to 0.55 in the PhIP-treated group in experiment 2 (data not shown). There was no statistically significant difference between PhIP-treated and control rats, and the values for PhIP-treated rats tended to be lower than those for the controls throughout the experiment. The tumor induction was not associated with any chronic increase in cell proliferation in background tissue, as evidenced by the BrdU labeling.

Table II. Incidence Data for Mammary Tumors of Different Histopathological Types in Rats Given PhIP for 48 Weeks

PhIP dose (ppm)	No. of rats	Fibro-adenoma	Intraductal papilloma	Adenocarcinoma			
				Intraductal	Solid-tubular	Papillo-tubular	Total
Experiment 1							
0	20	1 (5.0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
25	21	0 (0)	0 (0)	0 (0)	0 (0)	1 (4.8)	1 (4.8)
100	20	1 (5.0)	1 (5.0)	0 (0)	0 (0)	5 (25.0)*	5 (25.0)*
200	18	0 (0)	0 (0)	1 (5.6)	1 (5.6)	12 (66.7)***	13 (72.2)***
Experiment 2							
0	20	0 (0)	1 (5.0)	0 (0)	0 (0)	0 (0)	0 (0)
12.5	20	1 (5.0)	0 (0)	0 (0)	0 (0)	2 (10.0)	2 (10.0)
50	20	1 (5.0)	0 (0)	0 (0)	1 (5.0)	7 (35.0)**	7 (35.0)**

*, **, ***; Significantly different from the control values at $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Table III. Distribution of Mammary Adenocarcinomas in Rats Given PhIP for 48 Weeks

PhIP dose (ppm)	No. of rats	No. of tumor-bearing rats	Location of mammary tumors					
			Cervical-thoracic			Abdominal-inguinal		
			Left	Right	Total	Left	Right	Total
Experiment 1								
0	20	0	0	0	0	0	0	0
25	21	1	0	0	0	1	0	1
100	20	5	3	6	9	3	3	6
200	18	13	9	5	14	2	7	9
Experiment 2								
0	20	0	0	0	0	0	0	0
12.5	20	2	0	0	0	1	1	2
50	20	7	3	1	4	2	1	3

In the present studies, although the incidence of tumors in the group treated with 50 ppm PhIP was almost the same as that in the 100 ppm group, the multiplicities of mammary tumors of tumor-bearing rats are rather different between these groups, i.e., about 7.6 and 12.4 tumors/tumor-bearing rat in the 50 ppm and 100 ppm PhIP-treated groups, respectively (data not shown; see Table II and Fig. 3). This observation is interesting from the viewpoint of PhIP dose dependency at very low dose.

It is well established that PhIP causes adduct formation in DNA[17–19] and genetic damage is considered to be an important mechanism in the generation of mammary gland cancers.[20] From the present results, no clear role of proliferation in normal-appearing tissue could be demonstrated, suggesting that PhIP is not highly toxic to the mammary gland, and its induction of tumors in this site is not dependent on any regenerative processes. However, general toxicity was suggested by the significant decrease in body weights observed at the termination of the experiment.

In conclusion, the present data provide further evidence of the potential risk of human mammary cancer development from ingestion of PhIP. The demonstration of the lack of any dose threshold down to the 12.5 ppm level is particularly important given the possibility of summation or synergism between different heterocyclic amines at low doses.[11] Thus, this environmentally significant group of compounds may be extremely important with regard to human neoplasia.

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