Jpn. J. Cancer Res. 87, 1029–1033, October 1996

Efficient Method for Rapid Induction of Aberrant Crypt Foci in Rats with 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine

Masako Ochiai, Hitoshi Nakagama, Masatoshi Watanabe, Yukiko Ishiguro, Takashi Sugimura and Minako Nagao

Carcinogenesis Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) induces aberrant crypt foci (ACFs) as well as colon cancer in F344 male rats. Conditions allowing rapid development of ACFs over a short period were investigated. F344 male rats were fed 400 ppm of PhIP·HCl in a low-fat diet (LF) for 2 weeks and then given a PhIP-free, high-fat diet containing PRIMEX (HF-PR) or safflower oil, or PhIP-free LF for 4 or 12 weeks. Rats fed HF-PR for 4 weeks gave the highest number of ACFs/rat (3.3) and their size in terms of aberrant crypts/ACF(2.7) was much larger than that obtained with conventional continuous feeding of PhIP for 25 weeks in the CE-2 diet. Therefore, 2 weeks of dietary exposure to 400 ppm of PhIP·HCl, followed by HF-PR for 4 weeks, is a practical and convenient method for obtaining ACFs. This protocol should be useful for studies of the early phase of colon carcinogenesis.

Key words: ACF — PhIP — High fat diet

Heterocyclic amines (HCAs), which are produced by cooking meat and fish, are unique as carcinogens because humans are exposed to them in the diet on a daily basis.[1–3] Although colon carcinogens were found to be rare among compounds examined in the National Toxicology Program in the United States,[4,5] five out of ten HCAs originally isolated from cooked meat and fish and examined for carcinogenicity in rats, can induce colon cancer.[6]

2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), the most abundant HCA in cooked food,[2,3,6] is one such agent causing colon cancer development in F344 male rats.[7] Thus, experimental animal systems using PhIP should provide appropriate models for human colon carcinogenesis.

Aberrant crypt foci (ACFs), which are considered to be possible precancerous lesions in the colon, have been detected in experimental animals after treatment with various colon carcinogens.[8,9] They are now used as a biomarker in assays for colon carcinogens or promoters, as well as preventive agents.[10] In most studies, dimethylhydrazine or its proximate metabolite, azoxymethane, has been used to induce ACFs in experimental animals. However, PhIP also induces ACFs,[11,12] and does so at higher frequencies in male F344 rats than in female rats, which are less susceptible to PhIP-induced colon carcinogenesis.[13] This provides support for the validity of ACFs as an end-point lesion for colon carcinogenesis in medium-term tests. Fats, and especially corn oil and safflower oil, which contain linoleic acid at high concentrations, have a promoting effect on colon carcinogenesis.[14] Continuous feeding of PhIP in a high-fat diet containing corn oil induced more ACFs than did PhIP in a low-fat diet (LF).[15]

In this study we established a method for obtaining ACFs efficiently within a short period, by feeding high-fat diet after PhIP treatment. For comparison purposes, PRIMEX and safflower oil were selected for study, the former being high in oleic acid, which does not promote colon carcinogenesis in rats,[16] in contrast to the latter which is rich in linoleic acid and exerts clear promoting effects.[14,17]

MATERIALS AND METHODS

Animals and diets Five-week-old male F344 rats were obtained from CLEA Japan (Tokyo) along with the CE-2 basal diet. AIN-93G, a LF, and two high-fat diets obtained by adding hydrogenated vegetable oil, PRIMEX (HF-PR), or safflower oil (HF-SO), so as to supply 59% fat-derived calories in both cases, were purchased from Dyets Inc. (Bethlehem, PA). The HF-SO diet was supplemented with vitamin E (50 IU/kg) to prevent the hemolysis which is caused by dietary linoleic acid concentrations of over 5%.[18] The contents of fat were 7% soybean oil in the LF case, 10% soybean oil and 23% PRIMEX with HF-PR, and 10% soybean oil and 23% safflower oil with HF-SO. The linoleic acid component of fat constituted 22% of the HF-SO diet.

PhIP·HCl was purchased from the Nard Institute (Osaka) and added to CE-2 or AIN-93G to give a concentration of 400 ppm.

Animal experiments After one week's acclimation to the housing environment and the basal diet (CE-2 or AIN-93G), rats were subjected to either a conventional continuous PhIP-exposure protocol or a short PhIP-exposure protocol (Fig. 1). In the conventional continuous protocol (Fig. 1A), rats were fed PhIP in CE-2 diet for

25 weeks. With the short PhIP-exposure protocol (Fig. 1B), the animals were divided into 6 groups (I to VI) and treated as depicted in Fig. 1B. AIN-93G (LF) was used as the basal diet. Animals in groups I–III were given the PhIP diet for 2 weeks, while those in groups IV–VI received LF. Then, groups I and IV were fed HF-PR, groups II and V received HF-SO, and groups III and VI were given LF for 4 or 12 weeks.

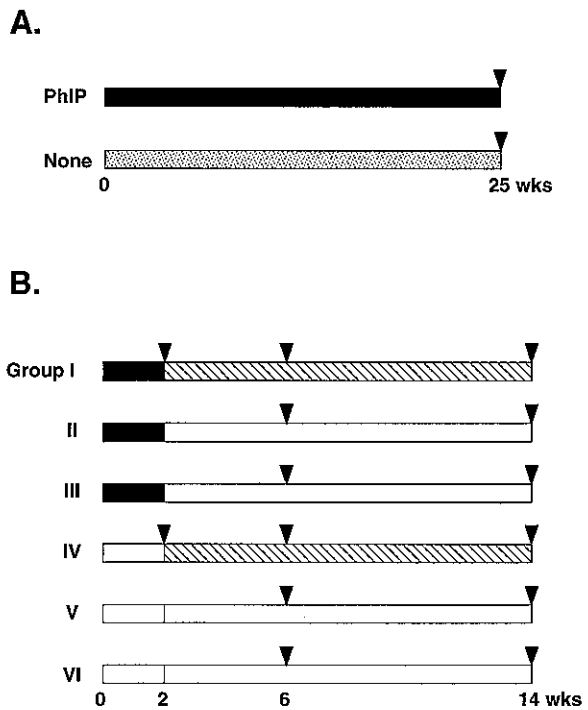

Fig. 1. Experimental protocols for the induction of ACFs by PhIP. A, Conventional continuous PhIP-exposure protocol. Rats were fed CE-2 diet containing 400 ppm of PhIP·HCl (■) or CE-2 diet alone (▒). B, Short PhIP-exposure protocol. Rats were subdivided into six groups, and fed either AIN-93G basal diet (LF) containing 400 ppm of PhIP·HCl (groups I–III, ■) or LF alone (groups IV–VI, □) for the first two weeks. Following this, the animals were fed PhIP-free HF-PR (groups I and IV, ▨), HF-SO (groups II and V, ▥) or LF (groups III and VI). At 6, 14 and 25 weeks, the numbers of rats indicated in Tables I and II were killed and analyzed for the induction of ACFs (▼). Five rats each in groups I and IV were also killed at the 2-week time point.

Detection of ACF Rats were killed after 2, 6, 14 or 25 weeks according to the protocols. The colons were removed and flushed out with neutralized 10% formalin, cut along the longitudinal median axis, submerged in formalin solution overnight, and then stained with 0.2% methylene blue as described by Bird.[8] The numbers of ACFs and aberrant crypts (ACs) were determined under 40× and 100× magnifications with a light microscope. The sizes of ACF were defined in terms of ACs/ACF.

Statistical analysis was performed using the Kruskal-Wallis one-way ANOVA and Mann-Whitney U tests, with an SPSS package on a Macintosh computer (SPSS Japan Inc., Tokyo).

RESULTS

Effect of PhIP on body weight change With the conventional continuous PhIP exposure protocol (Fig. 1A), the body weights of rats fed PhIP were about 75% of the control value after 25 weeks.

With the short PhIP-exposure protocol (Fig. 1B), the body weights of rats fed PhIP for 2 weeks (groups I–III) were also about 75% of those of animals not receiving PhIP (groups IV–VI). Recovery to about 90% of the untreated group value was noted after feeding a PhIP-free diet for 4 weeks, and the weights of treated and untreated rats essentially did not differ after 14 weeks. Although the food intake of rats in groups I (PhIP/HF-PR) and II (PhIP/HF-SO) was only around 80% of that of group III (PhIP/LF), the calorie intake of these 3 groups was almost the same, considering the calorie contents of each diet: HF-PR, 5016 kcal/kg; HF-SO, 5016 kcal/kg; LF, 3766 kcal/kg.

ACF induction by the conventional continuous PhIP exposure protocol After 25-week administration of PhIP in the CE-2 diet, ACFs were observed at a frequency of

Table I. ACFs Induced by the Conventional Continuous PhIP-exposure Protocol in Male F344 Rats

Chemical	Experimental period (wks)	Rats with ACFs	No./rat		No. of ACs/ACF	Ref.
			ACFs	ACFs of ≥4 ACs		
PhIP	14	7/7	6.6±1.5[a)]	0.4±0.8	1.6±1.2[a)]	13
	25	20/20	7.1±3.4[a*)]	0.1±0.3	1.6±0.8[a*)]	Present study
None	14	0/5	0	0	0	13
	25	0/5	0	0	0	Present study

a), *a**) Significantly different from that of rats fed CE-2 diet without supplement at the same time point[*a*), $P<0.01$; *a**), $P<0.001$].

Table II. ACFs Induced by the Short PhIP-exposure Protocol in Male F344 Rats

Chemical	Experimental group		Experimental period (wks)	Rat with ACF	No./rat		No. of ACs/ACF
					ACF	ACF of ≥4 ACs	
PhIP	I	HF-PR	6	6/6	$3.3\pm1.4^{a*)}$	$1.0\pm1.0^{a,b)}$	$2.7\pm1.8^{a*)}$
	II	HF-SO		5/6	$1.0\pm0.6^{a,c*)}$	0.2 ± 0.4	$2.0\pm1.1^{a)}$
	III	LF		5/6	$2.0\pm1.0^{a)}$	$0^{c)}$	$1.4\pm0.7^{a)}$
	I	HF-PR	14	4/5	$2.8\pm2.1^{a)}$	$1.4\pm1.4^{a)}$	$3.4\pm1.7^{a)}$
	II	HF-SO		6/6	$2.3\pm1.3^{a*)}$	$1.0\pm0.6^{a,d)}$	$4.0\pm2.4^{a*,d)}$
	III	LF		4/5	$1.8\pm1.0^{a)}$	0.4 ± 0.5	$2.9\pm1.8^{a,d)}$
None	IV	HF-PR	6	0/5	0	0	0
	V	HF-SO		1/5	0.2 ± 0.4	0	1.0
	VI	LF		0/5	0	0	0
	IV	HF-PR	14	1/5	0.2 ± 0.4	0	2.0
	V	HF-SO		0/5	0	0	0
	VI	LF		0/5	0	0	0

a), *a**) Significantly different from the value for rats of the same fat diet group without PhIP feeding at the same time point[*a*), $P<0.05$; *a**), $P<0.01$].
b) Significantly different from the value for rats fed LF at the same time point ($P<0.05$).
c), *c**) Significantly different from the value for rats fed HF-PR at the same time point [*c*), $P<0.05$; *c**), $P<0.01$].
d) Significantly different from the value for the experimental period of 6 weeks ($P<0.05$).

7.1/rat with an average size of 1.6 ACs/ACF. These values were almost the same as those obtained earlier with 14-week administration of PhIP,[13] where the frequency was 6.6 ACFs/rat and the size was 1.6 ACs/ACF (Table I). No ACFs were detected in control rats at week 14[13] or 25.

Comparison of ACF induction by the short and conventional continuous PhIP-exposure protocols With the short PhIP-exposure protocol, ACFs were detected in all PhIP-treated groups (I, II and III) after the experimental periods of 6 and 14 weeks (Table II). The numbers of ACFs in these groups were lower than that observed in the conventional continuous PhIP-exposure experiment. However, the average sizes of ACFs in PhIP-treated groups I, II and III after the experimental period of 14 weeks were much larger than that achieved with the conventional continuous PhIP-exposure protocol (Tables I and II).

Effects of high-fat diets on ACF development with the short PhIP-exposure protocol At the 6-week time point, rats in group I (PhIP/HF-PR) had the largest ACFs and the highest number of ACFs. At week 14, numbers of ACFs/rat and their sizes were almost the same in groups I (PhIP/HF-PR) and II (PhIP/HF-SO), and higher than in group III (PhIP/LF) (Table II).

In the control groups (IV–VI), only one ACF was detected in group V (HF-SO) at week 6 and one in group IV (HF-PR) at week 14.

The size distributions of the ACFs observed in groups I–III are depicted in Fig. 2. At week 6, ACFs composed

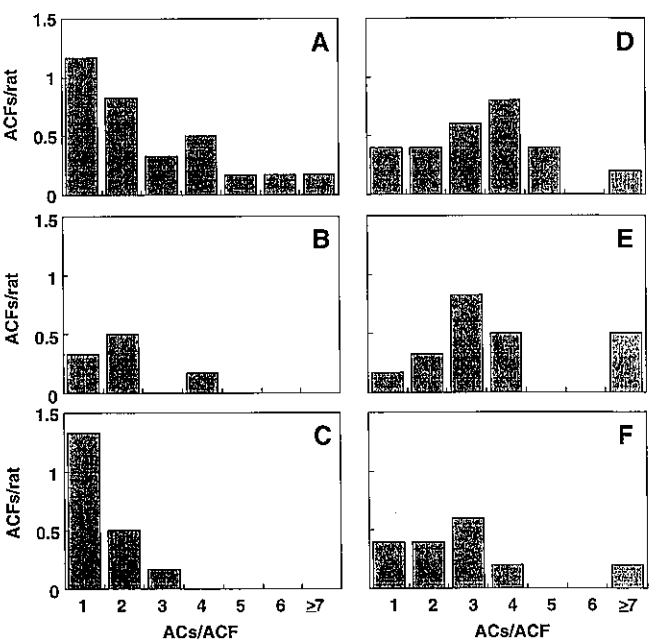

Fig. 2. Size distributions of ACFs as a function of time. ACFs were subgrouped according to their sizes, from 1 AC/ACF to more than 7 ACs/ACF and the numbers falling into each subgroup were counted to give average ACFs/rat values. Data for 6 weeks (A to C) and 14 weeks (D to F) are shown. A and D, PhIP/HF-PR; B and E, PhIP/HF-SO and C and F, PhIP/LF. The numbers of rats used for this analysis are given in Table II.

of only one AC were most frequent in groups I (PhIP/HF-PR) and III (PhIP/LF), although in the former case the size range was extensive, with some lesions even having seven or more ACs. In group II (PhIP/HF-SO) the 2 ACs/ACF category predominated (Fig. 2, A–C). At week 14, the numbers of ACFs composed of one AC were decreased in all groups and ACFs composed of 3 or 4 ACs were the most frequent (Fig. 2, D–F).

DISCUSSION

In the present study, we established a method for inducing large numbers of ACFs of relatively large size within only a 6-week experimental period using a protocol featuring 2 weeks of exposure to 400 ppm PhIP·HCl followed by 4 weeks on an HF-PR diet. Rats in this group had the highest number of lesions composed of 4 or more of ACs at 6 weeks, such large ACFs showing the best correlation with colon tumor incidence.[19] Since ACFs were rare (0.2 ACF/rat and 1.0 AC/ACF) at the end of the 2-week exposure to PhIP, development was primarily during the HF-PR feeding period. In other researchers'[12] and our[13] previous studies, continuous feeding of F344 rats with 400 ppm of PhIP·HCl in the diet for 8 or 14 weeks induced similar or larger numbers of ACFs, but their sizes were much smaller. Moreover, prolongation of the exposure period to a total of 25 weeks in the present study did not increase the number or size of ACFs (Table I). Thus, continuous feeding of PhIP does not appear to be the most appropriate method for obtaining biologically significant large ACFs efficiently within a short period. PhIP may possibly act as an inhibitor of ACF growth, or an inducer of apoptosis, even while initiating cells. Short exposure to PhIP followed by conditions conducive to rapid growth of the initiated cells is far better for this purpose. Guo *et al.*[20] obtained 3.3 ACFs/rat with a size of 3.4 ACs/ACF, when they gave PhIP (50 mg/kg, alternate days) by oral gavage for 2 weeks, followed by a PhIP-free diet for 12 weeks. We have obtained a similar size and number of ACFs in only 6 weeks.

Safflower oil, which is known to be a colon tumor promoter, did not show clear promoting effects under the conditions of this study. This may be due to the addition of vitamin E, which is known to inhibit progression to large ACFs in the rat.[21]

The protocol established in this study should find application in assays for tumor-promoting agents in colon carcinogenesis, and will also be a useful approach for elucidating genetic susceptibility to colon carcinogenesis.[22–24] Phenotyping of animals with regard to differences in ACF induction over a short period is an important next step for such studies.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan, a Grant-in-Aid for Aging and Health, and a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan.

(Received June 7, 1996/Accepted July 22, 1996)

REFERENCES

1) Nagao, M., Wakabayashi, K., Ushijima, T., Toyota, M., Totsuka, Y. and Sugimura, T. Human exposure to carcinogenic heterocyclic amines, and their mutational fingerprints in experimental animals. *Environ. Health Perspect.*, **104** (Suppl. 3), 497–501 (1996).
2) Stavric, B. Biological significance of trace levels of mutagenic heterocyclic aromatic amines in human diet: a critical review. *Food Chem. Toxicol.*, **32**, 977–994 (1994).
3) Layton, D. W., Bogen, K. T., Knize, M. G., Hatch, F. T., Johnson, V. M. and Felton, J. S. Cancer risk of heterocyclic amines in cooked foods: an analysis and implications for research. *Carcinogenesis*, **16**, 39–52 (1995).
4) Tennant, R. W. and Ashby, J. Classification according to chemical structure, mutagenicity to Salmonella and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U. S. National Toxicology Program. *Mutat. Res.*, **257**, 209–227 (1991).
5) Ashby, J. and Tennant, R. W. Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U. S. NTP. *Mutat. Res.*, **257**, 229–306 (1991).
6) Nagao, M. and Sugimura, T. Carcinogenic factors in food with relevance to colon cancer development. *Mutat. Res.*, **290**, 43–51 (1993).
7) Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayama, S. and Sugimura, T. A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). *Carcinogenesis*, **12**, 1503–1506 (1991).
8) Bird, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151 (1987).
9) Tudek, B., Bird, R. P. and Bruce, W. R. Foci of aberrant crypts in the colons of mice and rats exposed to carcinogens associated with foods. *Cancer Res.*, **49**, 1236–1240 (1989).
10) Bird, R. P. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett.*, **93**, 55–71 (1995).
11) Takahashi, S., Ogawa, K., Ohshima, H., Esumi, H., Ito, N.

and Sugimura, T. Induction of aberrant crypt foci in the large intestine of F344 rats by oral administration of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Jpn. J. Cancer Res.*, **82**, 135–137 (1991).

12) Hasegawa, R., Sano, M., Tamano, S., Imaida, K., Shirai, T., Nagao, M., Sugimura, T. and Ito, N. Dose-dependence of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) carcinogenicity in rats. *Carcinogenesis*, **14**, 2553–2557 (1993).
13) Ochiai, M., Watanabe, M., Kushida, H., Wakabayashi, K., Sugimura, T. and Nagao, M. DNA adduct formation, cell proliferation and aberrant crypt focus formation induced by PhIP in male and female rat colon with relevance to carcinogenesis. *Carcinogenesis*, **17**, 95–98 (1996).
14) Reddy, B. S. and Maeura, Y. Tumor promotion by dietary fat in azoxymethane-induced colon carcinogenesis in female F344 rats: influence of amount and source of dietary fat. *J. Natl. Cancer Inst.*, **72**, 745–750 (1984).
15) Weisburger, J. H., Rivenson, A., Hard, G. C., Zang, E., Nagao, M. and Sugimura, T. Role of fat and calcium in cancer causation by food mutagens, heterocyclic amines. *Proc. Soc. Exp. Biol. Med.*, **205**, 347–352 (1994).
16) Hogan, M. L. and Shamsuddin, A. M. Large intestinal carcinogenesis. I. Promotional effect of dietary fatty acid isomers in the rat model. *J. Natl. Cancer Inst.*, **73**, 1293–1296 (1984).
17) Sakaguchi, M., Hiramatsu, Y., Takada, H., Yamamura, M., Hioki, K., Saito, K. and Yamamoto, M. Effect of dietary unsaturated and saturated fats on azoxymethane-induced colon carcinogenesis in rats. *Cancer Res.*, **44**, 1472–1477 (1984).
18) Bieri, J. G. Kinetics of tissue α-tocopherol depletion and repletion. *Ann. NY Acad. Sci.*, **203**, 181–191 (1972).
19) Pretlow, T. P., O'Riordan, M. A., Somich, G. A., Amini, S. B. and Pretlow, T. G. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. *Carcinogenesis*, **13**, 1509–1512 (1992).
20) Guo, D., Schut, H. A. J., Davis, C. D., Snyderwine, E. G., Bailey, G. S. and Dashwood, R. H. Protection by chlorophyllin and indole-3-carbinol against 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-induced DNA adducts and colonic aberrant crypts in the F344 rat. *Carcinogenesis*, **16**, 2931–2937 (1995).
21) Shivapurkar, N., Tang, Z., Frost, A. and Alabaster, O. Inhibition of progression of aberrant crypt foci and colon tumor development by vitamin E and β-carotene in rats on a high-risk diet. *Cancer Lett.*, **91**, 125–132 (1995).
22) Shirai, T., Nakamura, A., Fukushima, S., Yamamoto, A., Tada, M. and Ito, N. Different carcinogenic responses in a variety of organs, including the prostate, of five different rat strains given 3,2′-dimethyl-4-aminobiphenyl. *Carcinogenesis*, **11**, 793–797 (1990).
23) Jacoby, R. F., Hohman, C., Marshall, D. J., Frick, T. J., Schlack, S., Broda, M., Smutko, J. and Elliott, R. W. Genetic analysis of colon cancer susceptibility in mice. *Genomics*, **22**, 381–387 (1994).
24) Moen, C. J. A., Groot, P. C., Hart, A. A. M., Snoek, M. and Demant, P. Fine mapping of colon tumor susceptibility (*Scc*) genes in the mouse, different from the genes known to be somatically mutated in colon cancer. *Proc. Natl. Acad. Sci. USA*, **93**, 1082–1086 (1996).