Jpn. J. Cancer Res. (Gann), 79, 691–697 ; June, 1988

Dose-dependent Induction of Liver and Thyroid Neoplastic Lesions by Short-term Administration of 2-Amino-3-methylimidazo[4,5-*f*]quinoline Combined with Partial Hepatectomy Followed by Phenobarbital or Low Dose 3′-Methyl-4-dimethylaminoazobenzene Promotion

Hiroyuki TSUDA,[*1] Makoto ASAMOTO,[*1] Tadashi OGISO,[*1] Tadashi INOUE,[*1] Nobuyuki ITO[*1] and Minako NAGAO[*2]

[*1]*First Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467 and* [*2]*Carcinogenesis Division, National Cancer Center Research Institute, 1-1 Tsukiji, 5-Chome, Chuo-ku, Tokyo 104*

Dietary administration of 0.1, 0.05, or 0.025% 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) for two weeks combined with partial hepatectomy at the end of the first week and followed by long-term treatment with phenobarbital (PB) or 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) from week 3 to week 86 resulted in dose-dependent development of liver and thyroid neoplastic and preneoplastic lesions. Quantitation of glutathione *S*-transferase placental form (GST-P)-positive hepatocellular focal populations revealed a significant correlation of IQ concentration with lesion area, with a yield approximately equal to that generated by a similar dose of 2-acetylaminofluorene. The fact that IQ was less toxic therefore allowed greater yields of hepatocellular carcinomas to be induced. The development of thyroid tumors initiated by the IQ treatment was significantly enhanced by the administration of PB, whereas Zymbal gland tumors induced by IQ did not show any correlation with either PB or 3′-Me-DAB treatment.

Key words: Pyrolysis products — Carcinogenicity — Liver — Thyroid — Dose dependency

Since the first demonstration of *in vivo* carcinogenicity of a tryptophan pyrolysis product in 1981[1] a number of the heterocyclic amines present in broiled foodstuffs have been shown to have tumorigenic potential in either mice or rats.[3–9] Thus, in addition to 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1)[1,2] and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-2),[3] glutamate pyrolysis products[5,6] and a number of heterocyclic imidazo derivatives[7–10] were all revealed to be potent carcinogens. These compounds have long been known to be mutagenic,[11] and low doses have furthermore been demonstrated to induce far greater yields when administered in combination than simple summation would predict[12] and therefore may be of direct and exceedingly important relevance to tumor development in man.

Abbreviations: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; PB, phenobarbital; 3′-Me-DAB, 3′-methyl-4-dimethylaminoazobenzene; GST-P, glutathione *S*-transferase placental form; 2-AAF, 2-acetylaminofluorene.

The compound 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) is a potent mutagen which was isolated from fish broiled under normal conditions.[13] It has been synthesized[14] and was reported to induce tumors in the forestomach, liver and lung of CDF1 mice[8] and liver, skin, Zymbal gland, colon and intestine of F344 rats.[7] Requiring microsome-dependent activation,[15] IQ has been demonstrated to be genotoxic in both bacteria and rat, hamster or guinea-pig hepatocytes in culture.[16,17] Since the earlier reports were based on data generated by long-term application, the present investigation was performed to ascertain whether limited exposure might prove effective for initiation. Partial hepatectomy was performed during the administration period and PB or a low level of 3′-Me-4-DAB was added in a second promotion stage to increase yields, as proposed previously.[18,19]

Materials and Methods

A total of 240 male 5-week-old F344 rats (Charles River Japan, Inc., Atsugi) housed 5 per

plastic cage with wood chips for bedding were maintained under constant conditions (12-hr light dark cycle, 60% humidity, 22°) on Oriental MF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. After one week for acclimation, animals were divided into groups as shown in the experimental regimen. Groups 1 to 3 received a two-week dietary treatment with IQ at doses of 0.1, 0.05 and 0.025%, respectively and were maintained on diet supplemented with PB (0.05%) or 3′-Me-DAB (0.0024%) from week 3 until final sacrifice at week 86. The dose of the latter was chosen as being itself insufficient for liver tumor development within the time scale of the experiments.[20] Group 4 served as a non-initiated control and group 5 received 0.1% IQ without subsequent promotion. IQ and 2-acetylaminofluorene (2-AAF) intake was calculated from 2-day consumption data measured biweekly. Groups 6 and 7 received 0.016% 2-AAF, this being the maximum dose allowing 100% survival after partial hepatectomy, with or without second stage promotion, and were maintained to give an approximate assessment of the comparative potency of IQ initiation. All animals underwent two-thirds partial hepatectomy at week 1.

Rats surviving till the end of the experiment and those dying of tumor development after week 52 were included in the effective numbers for assessment of neoplastic lesion yield by histopathological analysis of hematoxylin and eosin stained sections of formalin-fixed tissue from all major organs. In addition, at final sacrifice, liver slices were fixed in ice-cold acetone and paraffin sections cut and stained immunohistochemically for binding of specific antibodies to GST-P (generous gift of Dr. K. Sato, Hirosaki University), earlier shown to be an accurate marker for rat liver preneoplastic lesions[21] (raised as described previously[22]), using the avidin - biotin - peroxidase complex (ABC) method[23] (Vectastain Kit, Vector Laboratories Inc., Burlingame CA).

Incidence data were compared statistically using the χ^2 test whereas Student's t-test was applied for analysis of the significance of differences in areas of the GST-P-positive lesions.

Results

Effective numbers, and data on survival, mean body weights and IQ intake are summarized in Table I. Significant reduction in body weight as compared to appropriate controls was observed in all groups receiving IQ followed by PB or 3′-Me-DAB promoting regimens. Incidences of neoplastic lesions arising in the different groups are given in Tables II and III. Significant yields of hepatocellular carcinomas, thyroid lesions and Zymbal gland tumors were associated with initial IQ exposure. Yields were in general dose-related. In contrast, the development of preputial gland tumors appeared to be independent of IQ, similar incidences being observed in animals with or without the initiation treatment (see Table III). Occasional intestinal tumors were diagnosed but without any dose-dependence being apparent. PB exerted strong enhancing effects on development

Table I. Mean Survival Time, Body Weight and Total Initiator Intake

Group	Treatment		No. of rats	Mean survival time (days)	Mean body weight (g)	Total initiator intake (g/rat)
	Initiation by IQ/2-AAF (%)	Promotion by				
1	IQ (0.1)	PB	14	570.4±45.2[a]	313.1±67.1[c]	0.15
	IQ (0.05)	PB	18	583.2±29.0[a]	367.5±63.8[c]	0.09
	IQ (0.025)	PB	17	586.0±40.5	393.0±80.8[a]	0.05
	—	PB	18	602	447.3±34.6	0
2	IQ (0.1)	3′-Me-DAB	16	591.5±42.0	399.5±64.9[c]	0.15
	IQ (0.05)	3′-Me-DAB	18	582.4±51.8	415.4±69.7[b]	0.09
	IQ (0.025)	3′-Me-DAB	17	593.1±25.6	433.3±70.4[a]	0.05
	—	3′-Me-DAB	18	602	476.4±24.8	0
3	IQ (0.1)	—	19	586.5±38.5	404.0±61.1	0.15
4	2-AAF (0.016)	PB	18	596.8±21.9	422.8±40.5	0.03
5	2-AAF (0.016)	3′-Me-DAB	20	602	459.2±28.9	0.03
6	2-AAF (0.016)	—	17	602	449.3±22.1	0.03

Significantly different at $P<0.05$ (*a*), 0.01 (*b*), 0.001 (*c*) compared to the respective IQ dose 0 group.

Table II. Incidence and Area of Liver Lesions

Group	Treatment		No. of animals with (%)			Area of GST-P-positive lesion[a]	
	Initiation by IQ/2-AAF (%)	Promotion by	No. of rats	Hyperplastic nodules	Hepatocellular carcinomas	No. of rats[b]	mm²/cm²
1	IQ (0.1)	PB	14	14 (100)[e]	7 (50)[e,g]	8	29.91±10.52[e,g]
	IQ (0.05)	PB	18	18 (100)[e]	7 (38.9)[d,f]	10	33.58±14.29[e,g]
	IQ (0.025)	PB	17	17 (100)[e]	6 (35.3)[d,f]	10	18.93±6.88[e,g]
	—	PB	18	5 (29.4)	0 (0)	17	2.74±1.68
2	IQ (0.1)	3′-Me-DAB	16	16 (100)[e]	5 (31.3)[e,f]	14	14.07±4.23[e,g]
	IQ (0.05)	3′-Me-DAB	18	18 (100)[e]	3 (16.7)	13	12.78±4.35[e,g]
	IQ (0.025)	3′-Me-DAB	17	17 (100)[e]	1 (5.9)	14	8.95±3.82[e,g]
	—	3′-Me-DAB	18	7 (38.9)	0 (0)	18	1.65±0.79
3	IQ (0.1)	—	19	19 (100)	0 (0)	15	2.90±1.79
4	2-AAF (0.016)	PB	18	18 (100)	2 (11.1)	15	8.23±3.46[h]
5	2-AAF (0.016)	3′-Me-DAB	20	18 (90)	2 (10)	20	4.88±1.92
6	2-AAF (0.016)	—	17	14 (82.4)	0 (0)	17	4.58±2.78

a) Including hepatocellular carcinomas.
b) Survivors at week 86.
Significantly different at $P<0.05$ (*c*), 0.01 (*d*), 0.001 (*e*) compared to the respective IQ dose 0 group.
Significantly different at $P<0.01$ (*f*), 0.001 (*g*) compared to group 3.
Significantly different at $P<0.01$ (*h*) compared to group 6.

Table III. Incidence of Tumors in the Thyroid, Zymbal and Preputial Glands

Group	Treatment		No. of rats	No. of rats with (%)		
	Initiation by IQ/2-AAF (%)	Promotion by		Thyroid tumors[a]	Zymbal gland tumors[b]	Preputial tumors[c]
1	IQ (0.1)	PB	14	9 (64.3)[e,h]	6 (42.9)[e]	5 (35.7)
	IQ (0.05)	PB	18	8 (44.4)[d,g]	2 (11.1)	6 (33.3)
	IQ (0.025)	PB	17	8 (47.1)[d,g]	2 (11.8)	4 (23.5)
	—	PB	18	2 (11.8)	0 (0)	4 (22.2)
2	IQ (0.1)	3′-Me-DAB	16	3 (18.8)	1 (6.3)	6 (37.5)
	IQ (0.05)	3′-Me-DAB	18	2 (11.1)	0 (0)[f]	9 (50)
	IQ (0.025)	3′-Me-DAB	17	0 (0)	4 (23.5)[d]	5 (29.4)
	—	3′-Me-DAB	18	0 (0)	0 (0)	6 (33.3)
3	IQ (0.1)	—	19	1 (5.3)	5 (26.3)	7 (36.8)
4	2-AAF (0.016)	PB	18	2 (11.1)	0 (0)	1 (5.6)
5	2-AAF (0.016)	3′-Me-DAB	20	1 (5)	0 (0)	3 (15)
6	2-AAF (0.016)	—	17	0 (0)	0 (0)	3 (17.6)

a) Adenoma plus carcinoma.
b) Squamous cell carcinoma plus adenoacanthoma.
c) Adenoma, adenocarcinoma plus squamous cell carcinoma.
Significantly different at $P<0.05$ (*d*), 0.01 (*e*) compared to the respective IQ dose 0 group.
Significantly different at $P<0.05$ (*f*), 0.01 (*g*), 0.001 (*h*) compared to group 3.

of liver and thyroid tumors but not Zymbal gland lesions. Enhancement by 3′-Me-DAB appeared limited to the liver case.

Analysis of GST-P-positive lesions which included both foci and hepatocellular carcinomas (see Table II and Figs. 1 and 2) revealed

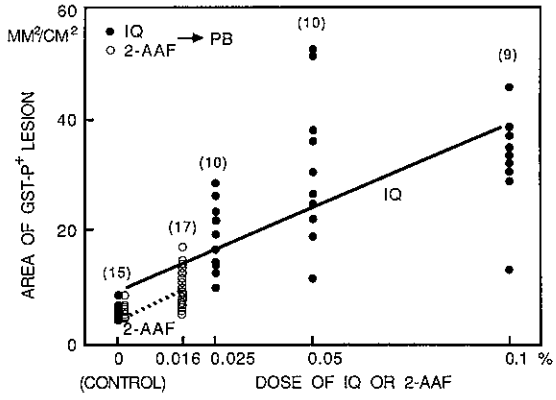

Fig. 1. IQ and 2-AAF dose dependences of GST-P-positive liver lesion development in animals promoted with PB. ( ), Number of rats, $r=0.743$, $P<0.001$, $Y=326X+7.85$ for IQ and $r=0.711$, $P<0.001$, $Y=331X+2.93$ for 2-AAF.

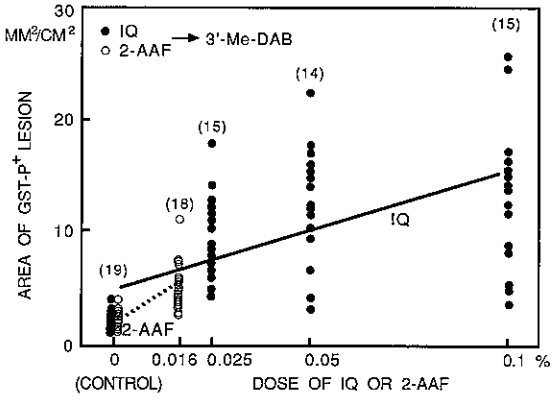

Fig. 2. IQ dose dependence of GST-P-positive liver lesion development in animals promoted with 3-Me-DAB. ( ), Number of rats. $r=0.614$, $P<0.001$, $Y=111X+4.45$ for IQ and $r=0.753$, $P<0.001$, $Y=202X+1.66$ for 2-AAF.

significant differences in area between groups with and without IQ administration. Both PB and 3′-Me-DAB alone brought about induction of a similar number of small lesions but PB appeared to exert a far greater promoting effect on the size of IQ- or 2-AAF-initiated foci and nodules. Indeed 3′-Me-DAB did not have any influence in the 2-AAF initiation case, and even with PB, the extent of enhancement was far less than after IQ initiation.

Thyroid lesions were almost all diagnosed as papillary adenomas and carcinomas, only a few cases of tumors with follicular morphology being observed. The Zymbal gland neoplasms were either squamous cell carcinomas or keratoacanthomas. The few intestinal tumors were adenomas or adenocarcinomas, whereas the preputial gland lesions were of squamous cell character.

Discussion

The results of the present investigation clearly demonstrated that even a relatively short administration of IQ given in combination with a regenerative stimulus to the liver can bring about a very effective initiation of hepatocarcinogenesis. The influence of partial hepatectomy is generally attributed to fixation of DNA lesions by cell division before repair processes can operate.[18, 19, 24, 25] However, the demonstrated high incidence of thyroid tumors would indicate that a proliferative stimulus, other than any inherent in possible carcinogen toxicity and associated regeneration, may be unnecessary for initiation of thyroid carcinogenesis by IQ. Investigation of the direct effects of IQ on thyroid tissue is warranted. This is, to the authors' knowledge, the first demonstration of the thyroid's susceptibility to IQ's carcinogenic potential, the earlier report of its effects in the rat being concerned with liver, Zymbal gland, colon, small intestine, skin, oral cavity and clitoral lesions.[7]

Strong initiating activities in rat liver were earlier demonstrated for medium-term treatment with other pyrolysis products, including lysine- and soybean globulin-derived compounds,[26, 27] and the present results thus suggest that all members of this group might share this potential. Indeed, the initiating potential of IQ was greater than that of 2-AAF at similar dose (see Figs. 1 and 2) although the level of toxicity was far lower, allowing application of far larger doses. In addition, it appears that at least IQ, and perhaps by analogy all these heterocyclic aromatic amines, also exerts a very pronounced promoting action for development of hepatocellular lesions. The extent of enhancement reported earlier was approximately equal to that observed for very strong hepatocarcinogenic regimens with 2-AAF, diethylnitrosamine,

and 3′-Me-DAB.[28] It should perhaps be stressed that the presence of IQ in the environment tends to imply that it is a direct relevance to human cancer, even though proof of an association is as yet lacking.[29] Therefore thorough testing of this important group of environmental contaminants in medium-term *in vivo* assay systems for both initiation and modulation activities is obviously warranted. The advantage of using enzyme markers for assessing dose-effect relationships, and thereby relative initiating and promoting potencies, has been emphasized by Pitot and his colleagues.[30] The marked effects of PB and to a lesser extent 3′-Me-DAB in the present experiment highlight the importance of post-initiation processes for tumor development. Indeed in extreme cases, a total shift from one tumor type to another may result from appropriate selection of promoting chemicals.[31] Since IQ is a multitarget carcinogen[5] the present results regarding development of only liver and thyroid tumors are in good agreement with the suggestion that manifestation of neoplastic lesions is heavily reliant on an appropriate promoting stimulus.

The fact that PB at the doses administered was far more effective at enhancing liver lesion development than was 3′-Me-DAB, even though both induced approximately equal numbers of nodules and small foci when given alone, is presumably an example of the variation in initiating and promoting potential. In view of the earlier demonstration that PB at the same dose as given in the present experiment strongly promoted development of DEN- and to a lesser extent aflatoxin B_1- but not N-OH-AAF-initiated lesions,[32] the observed finding of only a slight effect on 2-AAF-induced foci is of interest. Thus, in comparison to the 10-fold enhancement of IQ-initiated lesions by PB, the doubling of 2-AAF alone values is obviously low. Similarly, with 3′-Me-DAB the equivalent comparison is between a 5-fold increase and no effect whatsoever. Investigation of biochemical differences which presumably must underlie such variation in susceptibility to promotion is an as yet barely explored area.

Although a great deal of information has been generated as to the target organ specificity of different second-stage modulators of neoplastic development, again the mechanisms involved remain largely unclear.[33] The present finding of clear promotion of thyroid but not Zymbal gland tumors by PB is, however, in agreement with earlier reports.[34, 35]

Acknowledgments

This study was partly supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture and from the Ministry of Health and Welfare of Japan and by a Grant-in-Aid from the Ministry of Health and Welfare for a Comprehensive 10-Year Strategy for Cancer Control, Japan. The authors would like to express their gratitude to Dr. Kiyomi Sato, Second Department of Biochemistry, Hirosaki University School of Medicine, for providing GST-P antibody.

(Received March 3, 1988/Accepted April 25, 1988)

References

1) Matsukura, N., Kawachi, T., Morino, K., Ohgaki, H. and Sugimura, T. Carcinogenicity in mice of mutagenic compounds from a tryptophan pyrolysate. *Science,* **213,** 346 (1981).
2) Ishikawa, T., Takayama, S., Kitagawa, T., Kawachi, T., Kinebuchi, M., Matsukura, N., Uchida, E. and Sugimura, T. *In vivo* experiments on tryptophan pyrolysis products. *In* "Naturally Occurring Carcinogens-Mutagens and Modulators of Carcinogenesis," ed. E. C. Miller, J. A. Miller, I. Hirono, T. Sugimura and S. Takayama, pp. 159–167 (1981). Japan Scientific Societies Press, Tokyo/University Park Press, Baltimore.
3) Hosaka, S., Matsushima, T., Hirono, I. and Sugimura, T. Carcinogenic activity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Tr-P-2), a pyrolysis product of tryptophan. *Cancer Lett.,* **13,** 23–28 (1981).
4) Takayama, S., Nakatsuru, Y., Ohgaki, H., Sato, S. and Sugimura, T. Carcinogenicity in rats of a mutagenic compound, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole, from tryptophan pyrolysate. *Jpn. J. Cancer Res. (Gann),* **76,** 815–817 (1985).
5) Takayama, S., Masuda, M., Mogami, M., Ohgaki, H., Sato, S. and Sugimura, T. Induction of cancers in the intestine, liver and various other organs of rats by feeding mutagens from glutamic acid pyrolysate. *Gann,* **75,** 207–213 (1984).

6) Ohgaki, H., Matsukura, N., Morino, K., Kawachi, T., Sugimura, T. and Takayama, S. Carcinogenicity in mice of mutagenic compounds from glutamic acid and soybean globulin pyrolysates. *Carcinogenesis,* **5,** 815–819 (1984).
7) Takayama, S., Nakatsuru, Y., Masuda, M., Ohgaki, H., Sato, S. and Sugimura, T. Demonstration of carcinogenicity in F344 rats of 2-amino-3-methylimidazo[4,5-*f*]quinoline from broiled sardine, fried beef and beef extract. *Gann,* **75,** 467–470 (1984).
8) Ohgaki, H., Kusama, K., Matsukura, N., Morino, K., Hasegawa, H., Sato, S., Takayama, S. and Sugimura, T. Carcinogenicity in mice of a mutagenic compound, 2-amino-3-methylimidazo[4,5-*f*]quinoline, from broiled sardine, cooked beef and beef extract. *Carcinogenesis,* **5,** 921–924 (1984).
9) Ohgaki, H., Hasegawa, H., Suenaga, M., Sato, S., Takayama, S. and Sugimura, T. Carcinogenicity in mice of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline (MeIQx) from cooked foods. *Carcinogenesis,* **8,** 665–668 (1987).
10) Kato, T., Ohgaki, H., Hasegawa, H., Saito, S., Takayama, S. and Sugimura, T. Carcinogenicity in rats of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline. *Carcinogenesis,* **9,** 71–73 (1988).
11) Sugimura, T., Nagao, M., Kawachi, T., Honda, M., Yahagi, T., Seino, Y., Sato, S. and Matsukura, N. Mutagens-carcinogens in foods, with special reference to highly mutagenic pyrolytic products in broiled foods. *In* "Origins of Human Cancer," Book C, ed. H. H. Hiatt, J. D. Watson and J. A. Winstein, pp. 1561–1577 (1977). Cold Spring Harbor Laboratory, New York.
12) Takayama, S., Nakatsuru, Y. and Sato, S. Carcinogenic effect of the simultaneous administration of five heterocyclic amines to F344 rats. *Jpn. J. Cancer Res. (Gann),* **78,** 1068–1072 (1987).
13) Kasai, H., Yamaizumi, Z., Wakabayash, K., Nagao, M., Sugimura, T., Yokoyama, S., Miyazawa, T., Springarn, N. E., Weisburger, J. H. and Nishimura, S. Potent novel mutants produced by broiling fish under normal conditions. *Proc. Jpn. Acad.,* **56B,** 278–283 (1980).
14) Kasai, H., Nishimura, S., Wakabayashi, K., Nagao, M. and Sugimura, T. Chemical synthesis of 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ), a potent mutagen isolated from broiled fish. *Proc. Jpn. Acad.,* **56B,** 382–384 (1980).
15) Yamazoe, Y., Shimada, M., Kamataki, T. and Kato, R. Microsomal activation of 2-amino-3-methylimidazo[4,5-*f*]quinoline, a pyrolysate of sardine and beef extracts, to a mutagenic intermediate. *Cancer Res.,* **43,** 5768–5774 (1983).
16) Barnes, W. S., Lovelette, C. A., Tong, C., Williams, G. M. and Weisburger, J. H. Genotoxicity of the food mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and analogs. *Carcinogenesis,* **6,** 441–444 (1985).
17) Loury, D. J. and Byard, J. L. Genotoxicity of the cooked-food mutagens IQ and MeIQ in primary cultures of rat, hamster, and guinea-pig hepatocytes. *Environ. Mutag.,* **7,** 245–254 (1985).
18) Ishikawa, T., Takayama, S., Kitagawa, T., Kawachi, T. and Sugimura, T. Induction of enzyme-altered islands in rat liver by tryptophan pyrolysis products. *J. Cancer Res. Clin. Oncol.,* **95,** 221–224 (1978).
19) Kitagawa, T., Hirakawa, T., Ishikawa, T., Nemoto, N. and Takayama, S. Induction of hepatocellular carcinoma in rat liver by initial treatment with benzo(a)pyrene after partial hepatectomy and promotion by phenobarbital. *Toxicol. Lett.,* **6,** 167–171 (1980).
20) Ogiso, T., Tatematsu, M., Tamano, S., Tsuda, H. and Ito, N. Comparative effects of carcinogens on the induction of placental glutathione S-transferase-positive liver nodules in a short-term assay and of hepatocellular carcinoma in a long-term assay. *Toxicol. Pathol.,* **13,** 257–265 (1985).
21) Tatematsu, M., Mera, Y., Ito, N., Satoh, K. and Sato, K. Relative merits of immunohistochemical demonstrations of placental, A, B and C forms of glutathione S-transferase and histochemical demonstration of gamma glutamyltranspeptidase as markers of altered foci during liver carcinogenesis in rats. *Carcinogenesis,* **6,** 1621–1626 (1985).
22) Satoh, K., Kitahara, A., Soma, Y., Inaba, Y., Hatayama, I. and Sato, K. Purification, induction, and distribution of placental glutathione transferase: a new marker enzyme for preneoplastic cells in the rat chemical hepatocarcinogenesis. *Proc. Natl. Acad. Sci. USA,* **82,** 3964–3968 (1985).
23) Hsu, S. M., Raine, L. and Farger, H. Use of avidin-biotin-peroxidase complex (ABC) in immunohistochemical techniques: a comparison between ABC and unlabelled antibody PAP procedures. *J. Histochem. Cytochem.,* **29,** 577–580 (1981).
24) Ishikawa, T., Takayama, S. and Kitagawa, T. Correlation between time of partial hepatectomy after a single treatment with diethylnitrosamine and induction of adeno-

sine triphosphatase-deficient islands in rat liver. *Cancer Res.*, **40**, 4361–4364 (1980).

25) Hasegawa, R., Tsuda, H., Shirai, T., Kurata, Y., Masuda, A. and Ito, N. Effect of timing of partial hepatectomy on the induction of preneoplastic liver foci in rats given hepatocarcinogens. *Cancer Lett.*, **32**, 15–23 (1986).

26) Tamano, S., Tsuda, H., Tatematsu, M., Hasegawa, R., Imaida, K. and Ito, N. Induction of γ-glutamyl transpeptidase positive foci in rat liver by pyrolysis products of amino acids. *Gann*, **72**, 747–753 (1981).

27) Hasegawa, R., Tsuda, H., Ogiso, T., Ohshima, M. and Ito, N. Initiating activities of pyrolysis products of L-lysine and soybean globulin assessed in terms of the induction of gamma glutamyltranspeptidase-positive foci in rat liver. *Gann*, **73**, 158–159 (1982).

28) Ito, N., Tsuda, H., Tatematsu, M., Inoue, T., Tagawa, Y., Aoki, T., Uwagawa, S., Kagawa, M., Ogiso, T., Masui, T., Imaida, K., Fukushima, S. and Asamoto, M. Enhancing effect of various hepatocarcinogens on induction of preneoplastic glutathione S-transferase placental form positive foci in rats — an approach for a new medium term bioassay system. *Carcinogenesis*, **9**, 387–394 (1988).

29) IARC. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Vol. 40, 223–288 (1986). Oxford University Press, London.

30) Pitot, H. C., Goldworthy, T. L., Moran, S., Kennan, W., Glauert, H. P., Maronpot, R. R. and Campbell, H. A. A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose-response relationships to altered hepatic foci. *Carcinogenesis*, **8**, 1491–1500 (1987).

31) Nakanishi, K., Fukushima, S., Hagiwara, A., Tamano, S. and Ito, N. Organ-specific promoting effects of phenobarbital sodium and sodium saccharin in the induction of liver and urinary bladder tumors in male F344 rats. *J. Natl. Cancer Inst.*, **68**, 497–500 (1982).

32) Shirai, T., Imaida, K., Ohshima, M., Fukushima, S., Lee, M-S., King, C. M. and Ito, N. Different responses to phenobarbital promotion in the development of γ-glutamyltranspeptidase-positive foci in the liver of rats initiated with diethylnitrosamine, N-hydroxy-2-acetylaminofluorene and aflatoxin B_1. *Jpn. J. Cancer Res. (Gann)*, **76**, 16–19 (1985).

33) Ito, N. Organ-specific modifying effects of phenobarbital, saccharin and antioxidants on 2-stage chemical carcinogenesis. *In* "New Concepts and Developments in Toxicology," ed. P. L. Chambers, P. Gehring and F. Sakai, pp. 359–369 (1986). Elsevier Science Publishers B. V., Amsterdam.

34) Hiasa, Y., Kitahori, Y., Ohshima, M., Fujita, Y., Yuasa, T., Konishi, N. and Miyasato, A. Promoting effects of phenobarbital and barbital on development of thyroid tumors in rats treated with N-bis(2-hydroxypropyl)-nitrosamine. *Carcinogenesis*, **3**, 1187–1190 (1982).

35) Tsuda, H., Fukushima, S., Imaida, K., Kurata, Y. and Ito, N. Organ-specific promoting effect of phenobarbital and saccharin in induction of thyroid, liver, and urinary bladder tumors in rats after initiation with N-nitrosomethylurea. *Cancer Res.*, **43**, 3292–3296 (1983).