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Age-dependent Induction of Preneoplastic Liver Cell Foci by 2-Acetylaminofluorene, Phenobarbital and Acetaminophen in F344 Rats Initially Treated with Diethylnitrosamine

Ryohei Hasegawa,[1] Satoru Takahashi, Katsumi Imaida,[2] Shuji Yamaguchi, Tomoyuki Shirai and Nobuyuki Ito

First Department of Pathology, Nagoya City University Medical School, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467

Effects of age on the induction of glutathione *S*-transferase placental form (GST-P)-positive hepatic foci in rats were examined using a medium-term liver bioassay system (for carcinogens). F344 male rats aged 6, 26 and 46 weeks were initially given a single intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg) and, beginning 2 weeks later, received 0.02% 2-acetylaminofluorene (2-AAF), 0.05% phenobarbital (PB) or 1.3% acetaminophen (AAP) in the diet for 6 weeks. All animals were subjected to two-thirds hepatectomy 3 weeks after the DEN injection and were killed at week 8. Quantitative analysis of GST-P-positive foci revealed significantly ($P<0.001$) increased induction over control levels in terms of both numbers and areas for 2-AAF at all ages (6, 26 and 46 weeks), but especially in the 6-week-old case. In the PB- and AAP-treated groups, the respective enhancing and inhibitory influences were most pronounced in the animals aged 6 weeks, and were less marked in older rats. Thus, the response of F344 rats to the modifying effects of chemicals was age-dependent, the conclusion being drawn that young rats are more susceptible and therefore more appropriate for assessment of carcinogenic, promoting and inhibitory effects of chemicals.

Key words: Rat — Hepatocarcinogenesis — Medium-term bioassay — GST-P-positive foci — Age-dependent effect

It is well known that the response of animals to the carcinogenic action of chemicals differs between species, strains and sexes and with the manner of administration.[1)] Age is also a critical factor in the induction of tumors by chemical carcinogens[2)] and much evidence has been obtained suggesting that young animals are more susceptible than older ones to chemical carcinogens,[3–5)] although the reverse has also been reported.[6, 7)] In experimental liver carcinogenesis, it is well documented that rapidly regenerating hepatocytes after partial hepatectomy are more susceptible than cells in a static condition[8–11)] and newborns are usually more sensitive than older animals to single doses of chemicals.[12–14)]

In the present experiment, the influence of age on induction of glutathione *S*-transferase placental form (GST-P)-positive liver cell foci was investigated using a medium-term *in vivo* screening assay system for prediction of carcinogenicity of chemicals. The so-called DEN-PH model system has been developed as a compromise to overcome the disadvantages of *in vitro* short-term screening tests and long-term *in vivo* bioassays,[15, 16)] and the endpoint marker lesion used in the present study, GST-P-positive foci of the liver, has been established as the most reliable early marker in rat liver carcinogenesis.[16, 17)] 2-Acetylaminofluorene (2-AAF), a well-known strong liver carcinogen, phenobarbital (PB), a weak liver carcinogen or hepatopromoter,[18)] and acetaminophen (AAP) were used as test compounds. AAP has been shown to exert inhibitory effects on the induction of GST-P-positive foci[15, 19, 20)] and has no tumor-initiating[21)] or carcinogenic[22)] potential in rats, although it has been reported to be hepatocarcinogenic in one strain of mice.[23)]

MATERIALS AND METHODS

Animals and chemicals Male F344 rats were obtained from Charles River Japan Inc., Atsugi, at 5 weeks of age. They were housed, 5 to a cage, on wood-chip bedding in an air-conditioned animal room at 23 ± 2°C and 55 ± 5% humidity until use. Food (Oriental MF, Oriental Yeast Co., Tokyo) and water were available *ad libitum* throughout the experiment.

Diethylnitrosamine (DEN) and AAP were obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, 2-AAF from Nakarai Chemical Co., Kyoto, and PB from Iwaki Pharmaceutical Co., Tokyo. 2-AAF, PB and AAP were incorporated into powdered basal diet (Oriental MF) using a mixer at a dose of 0.02%, 0.05% and 1.3%,

[1] To whom all correspondence should be addressed.

[2] Present address: Division of Pathology, National Institute of Hygienic Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158.

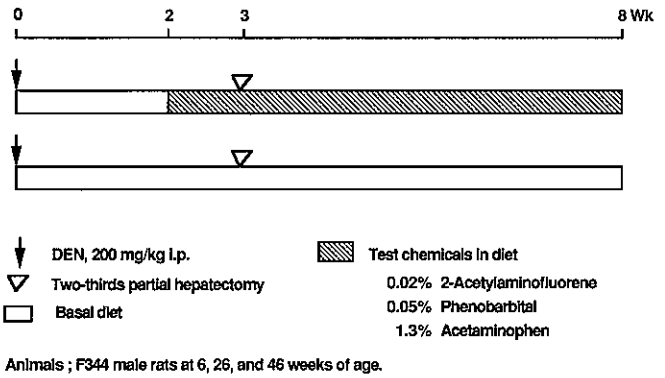

Fig. 1. Experimental design.

respectively, the diets being stored in a cold room until use.

Treatment and sampling of liver The experimental protocol is shown in Fig. 1. Groups of rats were initially intraperitoneally injected with DEN dissolved in saline at a dose of 200 mg/kg body weight when aged 6, 26 or 46 weeks. Starting 2 weeks later, the rats were maintained on powdered diet supplemented with one of the test chemicals or the basal diet as the control for 6 weeks. All rats were subjected to two-thirds partial hepatectomy (PH) at week 3. At the end of week 8, the rats were killed and the livers were immediately excised and sliced into sections of 2–3 mm thickness with a razor blade. Three slices, one from the caudate and two from the right anterior expanded lobes, were fixed in ice-cold acetone solution for immunohistochemical examination of GST-P expression. Additional slices were fixed in 10% phosphate-buffered formalin solution for routine staining with hematoxylin and eosin.

GST-P immunohistochemistry and analysis The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.*[24] was used to demonstrate GST-P-positive liver populations. After deparaffinization, liver sections were treated sequentially with normal goat serum, rabbit anti-GST-P (1:4000), biotin-labeled goat anti-rabbit IgG (1:400) and ABC. The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counter-stained with hematoxylin for microscopic examination. As a negative control for the specificity of anti-GST-P antibody binding, pre-immune rabbit serum was used instead of antiserum.

The numbers and the areas of GST-P-positive foci of more than 0.2 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (VIP-21C). The effects of test chemicals were assessed by comparing the number and area per unit area of liver section values for foci between test chemical-treated and basal diet groups in each age group. Statistical analyses were carried out using Student's *t* test and Welch's *t* test in combination with the *F*-test for variability.

RESULTS

The experiments with rats of 6 weeks old were performed separately for each chemical. Small numbers

Table I. Final Body and Liver Weights and Numbers and Areas of GST-P-positive Hepatocellular Foci

Age (weeks)	Group	No. of rats	Body weight (g)	Liver weight		GST-P-positive foci	
				Absolute (g)	Liver/Body (%)	No./cm^2	mm^2/cm^2
6	2-AAF	14	236±26***	12.51±2.79***	5.30±0.43***	65.47±11.25***	70.13±6.38***
	Basal diet	20	278±10	8.02±1.25	2.88±0.25	5.24±2.39	0.40±0.28
	PB	20	271±9**	10.62±0.91***	3.93±0.38***	10.46±2.66***	0.94±0.41***
	Basal diet	20	280±9	8.13±1.64	2.90±0.31	5.06±1.40	0.37±0.13
	AAP	22	248±20**	7.84±1.66	3.16±0.51*	2.59±1.08***	0.20±0.09***
	Basal diet	17	275±12	7.32±1.39	2.75±0.43	9.08±2.10	0.82±0.21
26	2-AAF	15	322±26***	9.37±1.55*	2.90±0.34***	37.57±5.79***	35.63±5.33***
	PB	10	357±24*	10.31±1.31***	2.88±0.22***	8.52±2.36	2.81±1.26*
	AAP	12	362±19*	8.84±0.68	2.44±0.08***	4.16±1.61**	1.10±0.57*
	Basal diet	9	386±24	8.32±0.64	2.16±0.11	7.13±2.34	1.78±0.77
46	2-AAF	5	392±28*	11.63±0.75***	2.97±0.06*	25.39±3.05***	29.71±4.46***
	PB	20	411±19	11.67±1.03***	2.84±0.18***	7.06±1.84*	1.40±0.46
	AAP	19	405±10*	10.17±0.78*	2.51±0.15*	2.51±1.20***	0.44±0.30***
	Basal diet	18	424±21	9.53±0.90	2.25±0.20	5.85±1.33	1.23±0.57

Significantly different from the appropriate control at: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

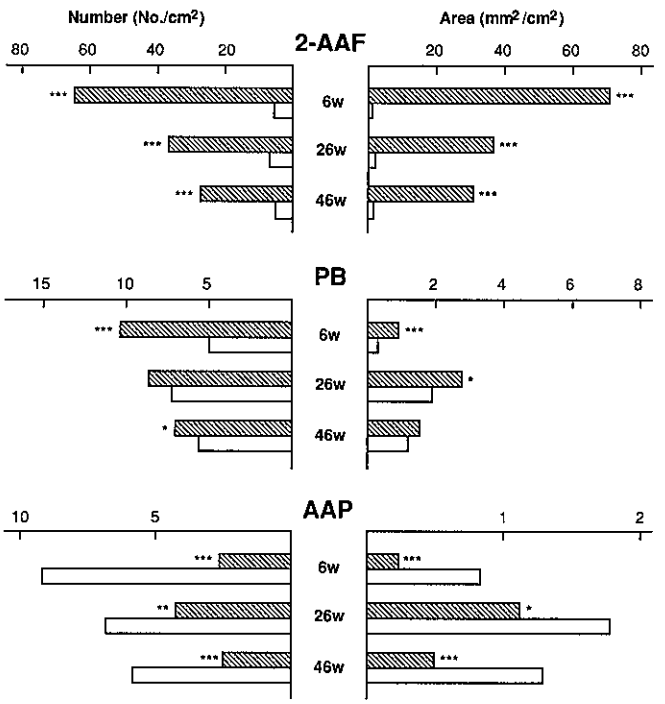

Fig. 2. Numbers and areas of GST-P-positive foci per unit area of liver section (cm^2). *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$ vs. the appropriate control groups.

of rats died during or after PH in all age groups and many died after PH in the 46-week group treated with 2-AAF.

Average body weights at the times of DEN injection were 140 ± 12, 380 ± 23, and 410 ± 17 for 6-, 26-, and 46-week-old animals, respectively. Body and liver weights at the end of experiment are summarized in Table I. Except for the 6-week-old group, in which rats grew rapidly, body weights of the control groups were almost the same as those at the time of DEN injection. In contrast, 2-AAF-treated groups showed marked lowering and PB- and AAP-treated groups, slight reduction in body weights as compared to the controls. Liver weights in all groups increased with age.

Data for numbers and areas of GST-P-positive foci per square cm of liver section observed are summarized in Table I, and also graphically illustrated in Fig. 2.

In the 6-week-old rats, 2-AAF and PB increased and AAP lowered both the numbers and areas of foci at statistically significant levels ($P<0.001$) as compared to the control values. 2-AAF was associated with a particularly marked increase. In rats aged 26 weeks, although the control level for area of foci was increased and absolute values for 2-AAF showed a reduction as compared to the 6-week group, highly significant differences were maintained. PB and AAP also showed enhancing and inhibitory effects, respectively, but at less pronounced levels of significance than in the 6-week-old cases. In rats aged 46 weeks, the control values were similar to those at 26 weeks, and whereas 2-AAF exerted a similar enhancing and AAP a more significant inhibitory influence, PB effects were less marked and only significant in terms of number.

DISCUSSION

The medium-term bioassay system (DEN-PH model) developed in our laboratory has various clear advantages over *in vitro* screening systems and long-term carcinogenicity studies of 2 years' duration, and has now been used to test more than 180 chemicals belonging to different categories, i.e. hepatocarcinogens, non-hepatocarcinogens and non-carcinogens, both genotoxic and non-genotoxic in the Ames assay. The details have been reported previously.[15, 16, 25] Trials using such a large number of chemicals have allowed the conclusion to be drawn that the system is reliable for general prediction of carcinogenic potency. The present experiment was conducted to determine the most appropriate age of rats for use in this system.

The comparison of male rats aged 6, 26, and 46 weeks regarding the modifying effects of 3 different test chemicals revealed age-dependent toxicity for DEN. In general, DEN and PH alone (control group) induced fewer, but larger, GST-P-positive foci in older animals, this being one reason why the enhancing effects of 2-AAF and PB were less marked in those cases. The inhibitory influence of AAP, on the other hand, seemed not to be as greatly affected by age. Xu *et al.*[26] reported a study in which both sexes of weanling, 6-month-old and 12-month-old rats were given a nonnecrogenic dose of DEN (10 mg/kg) 24 h after PH followed by PB promotion for 6 months and suggested that the observed lower level of altered liver cell foci in older rats might be due to less effective initiation and promotion by PB, since it is well known that the metabolism of some xenobiotics decreases with aging in both male and female rats.[27] It could be concluded that younger rats are more susceptible regarding both the initiation and promotion stages of carcinogenesis.

It is known that the eosinophilic foci, which are usually positive for GST-P immunoreactivity, spontaneously increase in older rats, especially in males,[28] and PB induces eosinophilic liver cell foci, even in females, much more quickly in old than in young rats.[29, 30] On the other hand, DEN-initiated lesions, as shown in the present study, exhibited a different response to PB, maximum enhancement being observed in 6-week-old animals. This is in agreement with an earlier report that male rats show

a dramatic reduction in the effectiveness of PB as a promoting agent at 12 months old as compared to weanling and 6-month-old animals.[26] Klaunig *et al.*[31] in fact found an inhibitory effect of PB on development of hepatocellular foci and adenomas in DEN-initiated mouse liver and, interestingly, the effect was more evident when the start of PB treatment was delayed.

2-AAF is a strongly genotoxic liver carcinogen and the age of the rats did not fundamentally affect the induction of GST-P foci in the present experiment. Using castrated male rats given testosterone, however, Reuber[32] reported that 2-AAF was a much more efficient carcinogen in rats at 12 weeks of age than in those at 52 weeks of age. This was postulated to be related to the fact that 2-AAF requires metabolic activation. Apparent toxicity of 2-AAF in the 46-week-old group observed in the present study may be strongly related to the prior DEN injection rather than the possible age-related 2-AAF metabolism since the DEN treatment was nearly lethal in rats of more than 1 year of age (unpublished data). AAP is a hepatotoxic agent which causes toxicity after metabolic activation in the liver.[33] The chemical has been reported as a liver carcinogen in one particular strain of mice,[23] but shows inhibitory effects in rat liver carcinogenesis as reported previously,[15, 19, 20] and confirmed in the present study. The influence of AAP, however, was age-independent.

In conclusion, the detection levels for tumorigenesis-modifying effects of the three chemicals tested were dependent to different extents on the age of the animal. Irrespective of the chemicals, effects could be more clearly detected in 6-week-old rats than in the older animals. It was earlier proposed that the present experimental protocol, which requires far fewer animals and shorter duration than a long-term carcinogenicity test, is advantageous for rapid screening of the large number of environmental chemicals which may have potential for induction of liver cancer in man. Based on the results of the present study, the use of male rats aged 6 weeks for our bioassay system is justified. This conclusion is also advantageous from the viewpoint of animal supply.

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