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Strain Differences in Susceptibility to 2-Acetylaminofluorene and Phenobarbital Promotion of Rat Hepatocarcinogenesis in a Medium-term Assay System: Quantitation of Glutathione *S*-Transferase P-positive Foci Development

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Strain differences in susceptibility to promotion by the liver carcinogens 2-acetylaminofluorene (2-AAF) and phenobarbital (PB) were examined in the medium-term bioassay system initially developed in our laboratory using male F344 rats as the test animal and glutathione *S*-transferase placental form (GST-P)-positive foci as the lesion end-point. Numbers and areas per cm^2 of induced GST-P-positive hepatocellular foci were compared in LEW, F344, NAR, SD, WBN, SHR, Wistar and ODS rats initiated with diethylnitrosamine (DEN) and subjected to partial hepatectomy during subsequent administration of 2-AAF or PB. LEW, SD, WBN, and F344 rats were most susceptible to hepatopromotion by both compounds, with a hundred fold increase in lesion area being observed for 2-AAF in the LEW case. NAR and SHR strains demonstrated an intermediate response, while Wistar and, in particular, the related ODS rats demonstrated very low susceptibilities. The obvious strain differences could be expressed in terms of comparative indices of promoting effects of 2-AAF and PB as well as DEN itself regarding each of the 8 strains tested. The use of F344 rats for the bioassay model was validated by the relatively high sensitivity to both DEN and 2-AAF initiation as well as second-stage promotion stimulus exhibited.

Key words: Strain difference — Hepatocarcinogenesis — Medium-term assay system — GST-P

Various experimental systems have been developed for the detection of environmental carcinogens or promoters.[1-4] Recently, we have concentrated our attention on developing an *in vivo* medium-term assay system using an F344 rat model combining partial hepatectomy with test compound administration following diethylnitrosamine (DEN[4])-initiation and quantitation of resultant liver preneoplastic foci.[5-9] A number of different markers have been used to visualize hepatocellular preneoplastic lesions[10,11] and in our laboratory, the glutathione *S*-transferase placental form (GST-P) has been demonstrated to be a very accurate marker enzyme[12-15]; it is hardly detectable in normal rat liver, but is very strongly expressed in preneoplastic liver lesions.[12,15] Using our system, the hepatocarcinogenic potential of 112 different compounds was checked, and of the liver carcinogens, 10 out of 11 mutagenic (90.1%), and 11 out of 13 non-mutagenic (84.6%) compounds gave positive results (mean 87.5%).[9]

Comparing the carcinogenicity of chemicals in different strains and species of animals is important for an understanding of potential hazard to man. Genetic factors exert a strong influence on the development of cancers,[16-20] and therefore we investigated different rat strains for susceptibility to the potent hepatocarcinogen 2-acetylaminofluorene (2-AAF) and the strong hepatopromoting agent phenobarbital (PB) in our medium-term assay system. F344, Wistar, ODS, SHR, LEW, WBN, SD and NAR strain rats were chosen for the study.

MATERIALS AND METHODS

Male rats of the following strains were used in the experiment: F 344 (Charles River Japan, Inc., Kanagawa), Wistar (Aburabi Lab., Shionogi Pharmaceutical Co., Ltd., Osaka), osteogenic disorder rat (ODS) (Aburabi Lab., Shionogi Pharmaceutical Co., Ltd.), spontaneous hypertensive rat (SHR) (Charles River Japan, Inc.), Lewis (LEW) (Charles River Japan, Inc.), WBN (WBN) (Shizuoka Laboratory Animal Center, Hamamatsu), Sprague-Dawley (SD) (Shizuoka Laboratory Animal Center) and Nagase analbuminemic (NAR) (Sasaki Institute, Tokyo). All rats were approximately 6 weeks of age at the start of the experiment and were housed in plastic cages in an air-conditioned room at 24±2°C. Animals of each strain were divided into three groups: group 1 was given a single intraperitoneal

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[4] Abbreviations: 2-AAF, 2-acetylaminofluorene; DEN, diethylnitrosamine; PB, phenobarbital; GST-P, glutathione *S*-transferase placental form; PH, partial hepatectomy.

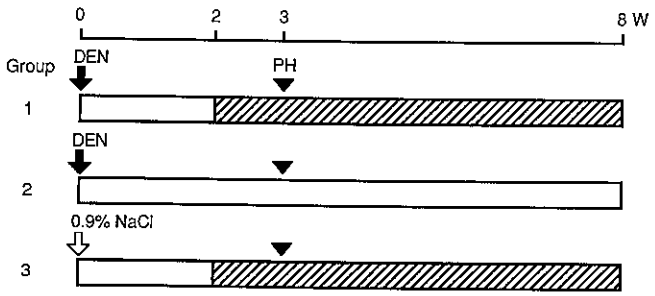

Fig. 1. Experimental design of the medium-term bioassay system used. Group 1, DEN + 2-AAF or PB; group 2, DEN alone; group 3, 2-AAF or PB alone. All rats were subjected to PH at week 3. DEN: 200 mg/kg, ip; PH: 2/3 partial hepatectomy; ▨: 0.02% 2-AAF or 0.05% PB in diet; animals: 6-week-old male rats.

injection of DEN (200 mg/kg) dissolved in 0.9% NaCl to initiate hepatocarcinogenesis, and then after 2 weeks on basal diet, received 0.02% 2-AAF or 0.05% PB in the powder diet (Oriental M, Oriental Yeast Co., Tokyo). All rats were subjected to partial hepatectomy (PH) at week 3. Each 2-AAF or PB group comprised 15 rats. Group 2 animals were given DEN and PH alone in the same manner, whereas group 3 rats were injected with 0.9% NaCl instead of DEN solution and then subjected to administration of 2-AAF or PB as with group 1, and PH (see Fig. 1). Immediately upon killing of the rats under ether anesthesia at week 8, the livers were removed and sections 2 to 3 mm thick were cut with a razor blade. Three slices, one each from the right lateral cranial, the right lateral caudal, and the caudate lobes, were fixed in 10% phosphate-buffered formalin solution for subsequent immunohistochemical examination of GST-P and routine staining with hematoxylin and eosin.

Anti GST-P antibody was raised as described previously.[13] The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.*[21] was used to determine the location of GST-P binding in the liver. Affinity-purified biotin-labeled goat anti-rabbit immunoglobulin IgG and avidin-biotin-peroxidase complex (Vectastain ABC Kit, PK 4001) were obtained from Vector Laboratories Inc. (Burlingame, CA). Paraffin sections were routinely passed through petroleum benzin and a graded alcohol series and then treated sequentially with normal goat serum, rabbit anti GST-P (1:8000), 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, preimmune rabbit serum was used instead of antiserum.

The numbers and areas of GST-P-positive foci of over 0.2 mm in diameter were measured using a color video image processor (VIP-21C, Olympus-Ikegami Tsushin Co., Tokyo) as described previously.[14, 15] The results were assessed by comparing the quantitative values between group 1 and group 2. Group 3 served to assess the carcinogenic potential of test compounds for induction of GST-P-positive foci without DEN initiation.

Statistical analyses were performed using Student's t test.

RESULTS

The numbers and areas of GST-P-positive foci for groups 1 to 3 in each of the 8 strains of rats tested are summarized in Tables I and II, respectively. After DEN initiation and PH without administration of 2-AAF or PB, the largest numbers and areas were observed in ODS and Wistar rats, the LEW, SD and WBN strains being relatively resistant. No foci were observed in PB-treated animals without DEN initiation. In contrast, 2-AAF alone induced large numbers of GST-P-positive lesions in LEW, WBN and F344 rats; in this case, ODS and Wistar strains demonstrated a relative lack of susceptibility.

Consideration of the comparative effects of PB or 2-AAF after DEN initiation was made by subtracting lesions induced by the respective individual treatments and dividing the resultant data for number and area by the relevant figures for DEN alone or 2-AAF alone (Table III). Thus, comparative values for promotion potency were generated for each strain and the relationship between carcinogenic and promotion potentials of 2-AAF could be examined.

For both compounds, promoting influence was most marked in LEW, SD, WBN and F344 strains. Thus, the degree of promotion by 2-AAF was highest in LEW rats with an enhancement factor of 1500 being observed for area and a factor of 80 for number of GST-P-positive lesions. SD, F344 and WBN also demonstrated very strong effects of 2-AAF whereas Wistar and the related ODS strains appeared resistant to hepatopromotion by this carcinogen.

No direct relationship between degree of induction of lesions by 2-AAF alone and extent of promotion of DEN-initiated lesion was apparent in data corrected for DEN alone (Table III). For example in the Wistar case, although the index of promotion was low at 10.47 for area, the almost total lack of lesions in the 2-AAF-alone group resulted in a high promotion-carcinogenicity ratio. On the other hand, in F344 rats a relatively high promotion potential was observed but the promotion-carcinogenicity index was low because 2-AAF itself induced many lesions.

Table I. No. of GST-P-positive Foci in the Liver (No./cm^2)

Strain	DEN	PB	2-AAF	DEN+PB	DEN+2-AAF
LEW	0.62±0.46	0	18.27±4.80	3.69±1.24***	70.46±18.06***
SD	1.30±0.50	0	3.93±2.83	4.52±2.10***	35.32±8.04***
NAR	3.08±1.66	0	3.21±1.99	7.23±2.77***	41.19±17.63***
ODS	12.77±3.87	0	0.63±0.45	12.75±3.37	41.90±14.55***
Wistar	5.50±1.27	0	0.08±0.16	9.22±2.25***	25.60±6.90***
WBN	1.12±1.14	0	19.81±3.38	2.40±1.17*	39.62±15.00***
SHR	2.58±1.09	0	2.90±1.37	3.76±1.84*	32.83±3.87***
F344	5.06±1.40	0	24.79±11.14	10.46±2.66***	65.47±11.25***

Significantly different from DEN alone: * $P<0.05$, *** $P<0.001$.

Table II. Area of GST-P-positive Foci in the Liver (mm^2/cm^2)

Strain	DEN	PB	2-AAF	DEN+PB	DEN+2-AAF
LEW	0.05±0.07	0	2.49±1.31	0.23±0.10***	78.00±11.45***
SD	0.07±0.03	0	0.94±0.68	0.24±0.13***	31.93±9.10***
NAR	0.17±0.10	0	0.18±0.10	0.42±0.20***	3.52±1.98***
ODS	0.93±0.42	0	0.09±0.10	1.18±0.47*	7.26±3.51*
Wistar	0.38±0.13	0	0.01±0.02	0.88±0.33***	3.99±1.15***
WBN	0.08±0.07	0	7.77±4.02	0.21±0.11***	18.16±6.38***
SHR	0.23±0.12	0	0.28±0.17	0.37±0.18*	20.59±7.48***
F344	0.37±0.13	0	16.53±7.79	0.94±0.41***	70.13±6.38***

Significantly different from DEN alone: * $P<0.05$, *** $P<0.001$.

Table III. Comparison of Degree of Effects

	PB		2-AAF			
	Promotion index		Promotion index		Promotion-carcinogenicity ratio	
	[DEN+PB]−[PB] / [DEN]		[DEN+AAF]−[2-AAF] / [DEN]		[DEN+2-AAF]−[DEN] / [2-AFF]	
Strain	No.	Area	No.	Area	No.	Area
LEW	5.95	4.60	80.30	1501.20	3.82	26.51
SD	3.48	3.43	24.10	442.71	8.66	33.89
NAR	2.35	2.47	12.33	19.56	11.87	18.61
ODS	1.00	1.27	3.23	7.71	42.23	70.33
Wistar	1.68	2.32	4.64	10.47	251.25	361.00
WBN	1.97	2.63	10.38	129.88	1.94	2.33
SHR	1.46	1.60	11.60	88.30	10.43	72.71
F344	2.08	2.54	8.04	144.86	2.08	4.22

DISCUSSION

The present investigation revealed significant strain difference in susceptibility to liver carcinogens in a medium-term (8 week) bioassay system for hepatopromotion, using preneoplastic glutathione *S*-transferase placental-type positive rat liver foci as the end marker lesion. With the exception of the F344 rats, which responded strongly to both compounds, in general, those strains most susceptible to 2-AAF administration proved resistant to DEN. This presumably reflects differential influence of the various drug-metabolizing systems responsible for carcinogenic and toxic effects of nitrosamine and aromatic amine hepatocarcinogens. Although those strains which demonstrated high response

to 2-AAF carcinogenicity also proved susceptible to promotion, no direct correlation between these two effects was apparent, as illustrated by the marked variation in promotion-carcinogenicity ratio. While it is clear that the formula used is only a rough approximation since the 2-AAF-alone value reflects both initiation and promotion components, the differences were large enough to suggest that the mechanisms underlying 2-AAF carcinogenicity and its promotion of DEN lesion development might indeed differ. This is in line with earlier demonstrations of variation in initiation as opposed to promotion potential of different carcinogens.[5, 22, 23] The idea of an index to allow direct comparison of the magnitude of promotion has been introduced previously.[24]

Since the LEW strain, which proved markedly susceptible to 2-AAF and PB, is a model animal for allergic or autoimmune disease,[25, 26] the present result indicates that the immune system also may be involved in hepatocarcinogenesis.

The NAR mutant strain which lacks serum albumin and shows hyperlipemia, was discovered in SD stock by Nagase *et al.*[27] Development of tumors in the urinary bladder,[28, 29] kidney,[30] stomach[31] and mammary gland[32] of NAR has been studied after various carcinogenic exposures and higher susceptibilities in NAR than in the parent SD strain were demonstrated for urinary bladder carcinogenesis induced by administration of N-butyl-N-(4-hydroxybutyl)nitrosamine,[28] renal tumorigenesis induced by N-dimethylnitrosamine[30] and gastric tumors after N-methyl-N′-nitro-N-nitrosoguanidine treatment.[31] However, sensitivity to carcinogens was not always scored to be higher in NAR than in SD animals. For example, the incidences of bladder cancer after administration of N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide[29] or mammary tumors induced by 7,12-dimethylbenz[*a*]anthracene[32] were observed to be lower in the NAR strain. In the present study, it was similarly shown that the susceptibility of NAR to 2-AAF or PB after DEN administration in liver carcinogenesis is lower than that of SD.

The ODS strain of rats derived from Wistar/Shi,[33] has a hereditary defect in L-ascorbic acid-synthesizing ability which is controlled by a single autosomal recessive gene.[34, 35] Since this deficiency interferes with long-term maintenance of ODS rats, they received a 0.05% L-ascorbic acid supplement in the basal diet in this study. ODS rats proved most sensitive to DEN initiation. However their susceptibility to PB or 2-AAF promotion was very low. The underlying response is unclear but there are many reports concerning possible relationships between L-ascorbic acid and chemical carcinogenesis,[36–38] and it is therefore probable that its administration might have exerted a considerable influence in the present experiment. Although this question remains to be resolved, the ODS strain does offer a very useful model for investigation of the effects of ascorbic acid deficiency on short-term carcinogen exposure.

Similarly, the marked differential evident in results for the WBN, constitutionally diabetic, and parent Wistar strains might suggest that investigation of metabolic pathways in these animals might also be very fruitful.

In our laboratory in the past few years we have been using the F344 rat strain in approaches to the development of new medium-term bioassay systems. This study showed that although LEW is most susceptible to promotion by 2-AAF, it is resistant to DEN initiation. ODS responds strongly to DEN but not to 2-AAF. Wistar rats appear resistant to initiation by 2-AAF while responding strongly to its promoting influence. However, this was not the case for PB. In contrast, F344 rats are relatively susceptible to both DEN initiation and promotion by either PB or 2-AAF. This finding plus the earlier demonstration that the system using F344 provides useful information concerning the inhibitory potential of compounds such as anti-oxidants thus suggests that the use of F344 rats for the present bioassay model is validated.

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