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Synergistic Effects of Low-dose Hepatocarcinogens in Induction of Glutathione *S*-Transferase P-positive Foci in the Rat Liver

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The effects of combined administration of hepatocarcinogens at low doses on the development of glutathione *S*-transferase P-form (GST-P)-positive foci of rat liver were examined utilizing a bioassay model which consists of a single injection of diethylnitrosamine (DEN, 200 mg/kg, ip), two-thirds partial hepatectomy at week 3 and a 6-week administration of test compounds. The chemicals used, 2-acetylaminofluorene (2-AAF), 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), phenobarbital (PB), thioacetamide (TAA), N-ethyl-N-hydroxyethylnitrosamine (EHEN), benzo[*a*]pyrene (B[a]P), carbazole, and α-hexachlorocyclohexane (α-HCH) were incorporated in the diet, except for EHEN which was dissolved in the drinking water, at levels of 1/6 of the doses usually used. The combinations were: I) 2-AAF, 3′-Me-DAB, PB, TAA, EHEN and B[a]P, II) 2-AAF, 3′-Me-DAB and PB, III) TAA, EHEN and B[a]P, IV) 2-AAF, 3′-Me-DAB, carbazole, TAA, EHEN and α-HCH, V) 2-AAF, 3′-Me-DAB and carbazole, and VI) TAA, EHEN and α-HCH. All combinations, except for II, caused an increase in the area of the foci as evaluated by the ratios of areas in the combined administration groups to the sum totals of 3 or 6 individual data: I) 1.75, II) 0.81, III) 2.01, IV) 3.62, V) 1.34 and VI) 2.91. The non-synergistic effect in combination II might be related to PB induction of hepatic microsomal enzymes leading to enhanced enzymatical detoxification of 2-AAF and 3′-Me-DAB. The present results indicate that exposure to several chemicals of similar organotropism, even at doses lower than the apparent carcinogenic levels, might be critical to the carcinogenic process.

Key words: Synergism — Low-dose carcinogen administration — GST-P — Rat liver

Since most human cancers are thought to be caused by environmental carcinogens,[1-5] it is now recognized that detection and regulation of these compounds are of prime importance for management of human neoplasia. However, human populations, except in particular cases where individuals are treated with specific therapeutic agents, suffer from some viral infectious diseases which may have potential to cause cancers or are occupationally exposed to some carcinogenic or radioactive agents, may rarely be exposed to significant doses of carcinogenic agents.[6]

It has been well demonstrated that chemical carcinogens act summationally and/or synergistically in the carcinogenic process,[7-12] and recently the two-step or multi-step carcinogenesis concept has been established for many organ models including the liver.[13-15] Thus, cancer development may actually depend on exposure to several carcinogenic or modifying agents simultaneously or sequentially. For this reason, it has become increasingly important that combined effects of chemicals on development of tumors be assessed.

In the present experiment, we focused on liver carcinogenesis, utilizing the medium-term bioassay system which has been developed in our laboratory over the last few years.[16-18] The prime aim was to examine the effects of combined exposure to low doses of liver carcinogens during the promotion stage, and the chemicals used were all known to possess carcinogenic or promoting activity for the liver. For example, 2-acetylaminofluorene (2-AAF) is a well-known hepatotoxic agent as well as a strong hepatocarcinogen,[19,20] and 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) induces poorly differentiated hepatocellular carcinomas or tumors of ductular cell origin.[21] The other agents chosen were phenobarbital (PB), a well known hepatopromoter recently classified as a weak hepatocarcinogen,[22] thioacetamide (TAA), a hepatotoxic laboratory chemical shown to be carcinogenic in the liver,[23] N-ethyl-N-hydroxyethylnitrosamine (EHEN), which induces liver and kidney lesions,[24] benzo[*a*]pyrene (B[a]P), which is capable of causing liver tumors when given after PH,[25] and carbazole[26] and α-hexachlorocyclohexane (α-HCH),[27] which have both been demonstrated to be weakly hepatocarcinogenic in the mouse.

MATERIALS AND METHODS

Animals and chemicals A total of 295 male F344 rats were obtained from Charles River Japan Inc., Atsugi, and maintained on basal diet (Oriental M, Oriental Yeast

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Co., Tokyo) *ad libitum*. They were housed in plastic cages in an air-conditioned room at 24±2°C and 55±5% humidity. Diethylnitrosamine (DEN), 3′-Me-DAB, TAA, B[a]P, and α-HCH were obtained from Tokyo Chemical Industry Co., Ltd., Tokyo, 2-AAF, and EHEN from Nakarai Chemical Co., Osaka, PB from Iwaki Pharmaceutical Co., Tokyo, and carbazole from Wako Pure Chemical Industry Ltd., Osaka.

Treatment of animals The animals were divided into 3 groups as shown in Fig. 1. Group 1 was given a single intraperitoneal injection of DEN at a dose of 200 mg/kg dissolved in 0.9% NaCl solution to initiate hepatocarcinogenesis. After 2 weeks, the rats were divided into 14 sub-groups and each was given one of the test treatments for the following 6 weeks. Group 2 was given DEN and then maintained on basal diet as a negative control. Group 3, which consisted of 14 sub-groups, was treated with the test chemicals for 6 weeks beginning 2 weeks after intraperitoneal injection of the vehicle. In all groups, rats were subjected to two-thirds partial hepatectomy (PH) at week 3. The rats were given free access to diet and water. At the end of week 8 of the experiment, all surviving rats were killed under light ether anesthesia after overnight fasting. Food and water intake and body weights were periodically measured.

Combined treatments of rats with the test chemicals were as follows: I) 2-AAF, 3′-Me-DAB, PB, TAA, EHEN and B[a]P, II) 2-AAF, 3′-Me-DAB and PB, III) TAA, EHEN and B[a]P, IV) 2-AAF, 3′-Me-DAB, carbazole, TAA, EHEN and α-HCH, V) 2-AAF, 3′-Me-DAB and carbazole, and VI) TAA, EHEN and α-HCH. The chemicals except for EHEN, which was given in the drinking water, were incorporated into powdered basal diet together. Eight sub-groups receiving single test chemicals were also included for each DEN-initiated (group 1) and non-initiated group (group 3).

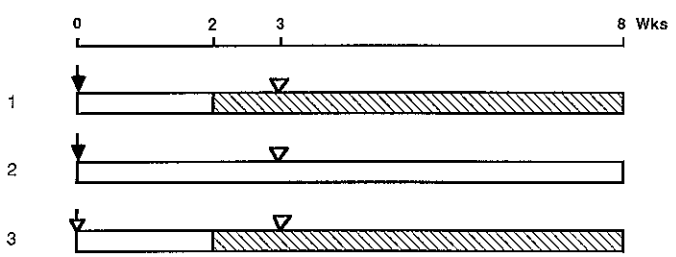

Fig. 1. Experimental design. Test chemicals used and their doses and combinations are listed in "Materials and Methods." Groups 1 and 3 consisted of 14 sub-groups with different test chemical administration. ↓ DEN, 200 mg/kg ip; ▽ two-thirds partial hepatectomy; ↓ saline, 2 ml/kg ip; ▨ test chemicals.

The doses and routes of administration of the test chemicals were as follows: 2-AAF, 33 ppm in diet; 3′-Me-DAB, 100 ppm in diet; PB, 83 ppm in diet; TAA, 100 ppm in diet; carbazole, 33 ppm in diet; EHEN, 83 ppm in drinking water; B[a]P, 33 ppm in diet; and α-HCH, 166 ppm in diet. The doses chosen were approximately 1/6 of the doses usually used in rat carcinogenicity studies. The diets were analyzed for each chemical 1 and 4 weeks after preparation, and no significant changes in concentration were evident.

Immediately upon killing of the animals, the livers were excised and weighed, and 2–3 mm thick slices were cut with a razor blade. Four slices, one each from the right and caudate lobes and two from the right anterior lobe, were fixed in ice-cold acetone for subsequent immunohistochemical staining for glutathione *S*-transferase placental form (GST-P). Additional slices were fixed in 10% phosphate-buffered formalin solution for routine staining with hematoxylin and eosin.

Immunohistochemical staining Anti-GST-P antibody was raised as described previously.[28] The avidin-biotin-peroxidase complex (ABC) method described by Hsu *et al.*[29] was used to determine the location of GST-P binding in the liver. Affinity-purified biotin-labeled goat anti-rabbit immunoglobulin IgG and ABC (Vectastain ABC kit, PK 4001) were obtained from Vector Laboratories Inc. (Burlingame, CA). Paraffin sections were routinely passed through graded alcohols and petroleum benzin, exposed to GST-P antibody (1:8000), biotin-labeled goat anti-rabbit IgG (1:400) and peroxidase complex. 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.

Quantitative analysis Numbers and areas of GST-P-positive foci of more than 0.2 mm diameter and total area of the liver sections examined were measured using a color video image processor (VIP-21C, Olympus-Ikegami Tsushin Co., Tokyo) as described previously.[16, 17, 30]

Quantitative analysis was performed using net values obtained by subtracting the background level (control value) from the measured areas for each sub-group. The effects of combined treatment with 3 or 6 chemicals on the development of GST-P-positive foci were assessed by comparing net values of the combined-treatment group with the sum totals of individual values for the chemicals used. Synergism was considered to have occurred when net values were significantly greater than the sum totals.

Statistical analysis for body and liver weights was carried out using Student's *t*-test and Cochran's *t*-test in combination with the *F*-test for variability.

RESULTS

Body and liver weight The body weights were generally lower in the DEN-initiated groups than in the non-initiated groups for each chemical treatment tested. Combined treatments with low-dose chemicals slightly retarded the body weight gain. In sub-groups treated with combined administration of carcinogens, body weights were slightly lower than in the controls (DEN-PH) except for combinations III) 2-AAF, 3′-Me-DAB, PB and V) 2-AAF, 3′-Me-DAB, carbazole, which showed similar weights to the control group in the DEN-initiated groups. Liver weight was most increased in the groups treated with α-HCH, solely or in combination with other chemicals. PB also increased the liver weight, but to a lesser extent than α-HCH.

Food intake was slightly lower in sub-groups treated with 6 chemicals together; 10.6 g/day in combination I and 11.8 g/day in combination IV as compared with 14.3–16.9 g/day in the single test chemical-treated sub-groups. Water intake was almost the same in all sub-groups (18–20 ml/day).

Quantitative analysis of GST-P-positive liver foci Net areas of GST-P-positive foci were increased by all the combined treatments, with the exception of combination II, when compared with the sum totals of those in single chemical groups (Table I).

Although all the test chemicals are hepatocarcinogens or promoters, groups treated with DEN injection prior to test chemical administration commonly exhibited higher levels of GST-P induction. In sub-group I, which was treated with 6 chemicals together (2-AAF, 3′-Me-DAB, PB, TAA, EHEN and B[a]P), the area of the foci (28.5 mm^2/cm^2) was 1.75 times higher than the sum total of data for the 6 individual chemicals (16.24 mm^2/cm^2: 9.39 +0.79+0.62+3.21+2.07+0.16) and 1.45 times higher than the total area of the 2 sub-groups treated with 3 chemicals each (19.68 mm^2/cm^2: 8.78+10.95). Similarly, the other sub-group treated with 6 chemicals (IV; 58.09 mm^2/cm^2) showed 3.62 and 1.90 times higher areas, respectively, than the sum total of the 6 individual (16.04 mm^2/cm^2) and 2 combined treatment (30.56 mm^2/cm^2) sub-groups. In the sub-groups treated with 3 chemicals, the areas of the foci were 1.34–2.91 times higher than the appropriate sum total areas, except for one combination (II) in which the situation was reversed: the combined treatment with 2-AAF, 3′-Me-DAB and PB resulted in slightly lower areas than the sum total (the ratio was 0.81). These results are illustrated in Fig. 2.

In sub-groups without DEN initiation, the combined effects of carcinogens were very marked, as shown in Table I. Areas of foci were as high as in DEN-initiated groups for combinations I, III, IV and VI. The ratios of the areas to the appropriate sum total data were more than 200 in combinations III, IV, and VI (TAA+EHEN + B[a]P, 2-AAF + 3′-Me-DAB + carbazole + TAA +

Table I. Area of GST-P-positive Liver Foci in Rats Treated with Low-dose Carcinogens

Treatment	DEN-initiation					Non-initiation				
	No. of rats	Measured area	Net (x)	Sum (y)	Ratio (x/y)	No. of rats	Measured area	Net (x)	Sum (y)	Ratio (x/y)
a+b	16	29.07±8.37	28.49	16.24	1.75	5	16.59±3.39	16.59	0.19	87
a	15	9.36±3.00	8.78	10.80	0.81	4	0.31±0.21	0.31	0.14	2.2
b	13	11.53±3.32	10.95	5.44	2.01	5	15.80±5.36	15.80	0.05	316
c+d	14	58.67±9.59	58.09	16.04	3.62	5	40.12±8.47	40.12	0.19	211
c	16	14.33±4.34	13.75	10.27	1.34	5	0.97±0.68	0.97	0.14	6.9
d	15	17.39±3.16	16.81	5.77	2.91	5	10.37±3.14	10.37	0.05	207
2-AAF	15	9.97±5.65	9.39			5	0.14±0.09	0.14		
3′-Me-DAB	14	1.37±0.35	0.79			4	0.00±0.01	0.00		
PB	14	1.20±0.55	0.62			4	0			
TAA	14	3.79±0.87	3.21			5	0.01±0.02	0.01		
EHEN	14	2.65±0.94	2.07			4	0.04±0.03	0.04		
B[a]P	15	0.74±0.30	0.16			4	0			
Carbazole	15	0.67±0.31	0.09			5	0			
α-HCH	15	1.07±0.42	0.49			4	0			
None	14	0.58±0.20	0			—				

Data presented are mean±SD values (mm^2/cm^2). a: 2-AAF+3′-Me-DAB+PB; b: TAA+EHEN+B[a]P; c: 2-AAF+3′-Me-DAB+carbazole; d: TAA+EHEN+α-HCH.

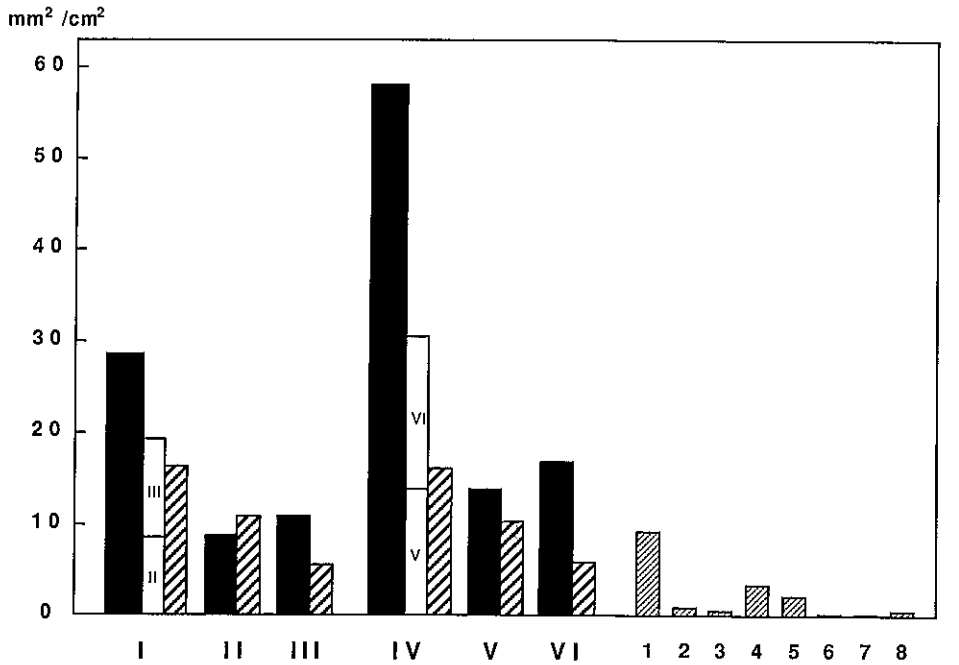

Fig. 2. Effects of combined treatment with several hepatocarcinogens after initiation with DEN on the induction of GST-P-positive rat liver foci (mm^2/cm^2). I) 2-AAF, 3′-Me-DAB, PB, TAA, EHEN and B[a]P, II) 2-AAF, 3′-Me-DAB and PB, III) TAA, EHEN and B[a]P, IV) 2-AAF, 3′-Me-DAB, carbazole, TAA, EHEN and α-HCH, V) 2-AAF, 3′-Me-DAB and carbazole, VI) TAA, EHEN and α-HCH, 1) 2-AAF, 2) 3′-Me-DAB, 3) PB, 4) TAA, 5) EHEN, 6) B[a]P, 7) carbazole, and 8) α-HCH. Net values were obtained by subtracting the control value (background level) from the measured area and used in the evaluation. ■: Combined treatment; □: sum total of 2 combined sub-groups; ▨: sum total of 3 or 6 individual data; ▨: individual treatment.

EHEN + α-HCH, and 2-AAF + 3′-Me-DAB + carbazole). The value of 2.2 was found even in combination II (2-AAF + 3′-Me-DAB + PB).

On the other hand, the numbers of foci were very high even in single chemical-treated sub-groups such as 2-AAF, TAA, and EHEN and no clear synergistic effects of the carcinogens were evident (Table II).

DISCUSSION

In the present study, several test chemicals were simultaneously administered to rats during the promotion stage after DEN initiation and areas of preneoplastic GST-P-positive liver foci were quantitatively examined. Practically, synergistic effects were evaluated by simply comparing results for areas of the combined-treatment groups with the sum totals of the 3 or 6 values obtained for single chemical treatment groups.

Takayama *et al.*[11] reported synergistic effects of five mutagenic and carcinogenic heterocyclic amines in F344 rats using doses set for each chemical at levels 1/5 of those used in previous carcinogenicity studies. As in many previous studies,[9,12] they assessed effects using only incidences of tumors and revealed synergism in the liver, colon, skin, and Zymbal glands. However, since we used only 15 rats for each sub-group, synergistic effects were evaluated based on quantitative values instead of incidence data in the present study. The end-point lesions we used in the present study were GST-P-positive liver foci, this enzyme marker being regarded as the most accurate presently available, with practical advantages over other markers.[28,31] This type of quantitative analysis may be more suitable for assessment than those utilizing incidence data since weak synergistic effects of chemicals are also detectable. The use of preneoplastic lesions also has the advantage of saving both animals and time.

Of the 6 combined administrations of 3 or 6 chemicals at low doses, five showed apparent synergistic activity on development of liver foci. The synergistic effect was, however, only detectable using the area of the GST-P-positive foci as the end-point, and not with the numbers

Table II. Number of GST-P-positive Liver Foci in Rats Treated with Low Doses of Carcinogens

Treatment	DEN-initiation		Non-initiation	
	No. of rats	No. of foci (No./cm^2)	No. of rats	No. of foci (No./cm^2)
a+b	16	71.2±13.9	5	86.2±6.2
a	15	49.1±5.8	4	4.0±2.1
b	13	78.7±16.2	5	97.6±7.6
c+d	14	27.5±15.4	5	66.0±16.0
c	16	53.4±7.7	5	9.4±5.2
d	15	84.9±7.6	5	82.3±8.4
2-AAF	15	52.7±5.9	5	2.2±1.5
3′-Me-DAB	14	16.9±2.8	4	0.1±0.2
PB	14	13.8±1.7	4	0
TAA	14	35.5±4.2	5	0.1±0.2
EHEN	14	29.4±7.4	4	1.0±0.8
B[a]P	15	9.5±2.0	4	0
Carbazole	15	7.7±3.0	5	0
α-HCH	15	13.3±4.2	4	0
None	14	8.2±2.9	—	

Data presented are mean±SD values. a: 2-AAF+3′-Me-DAB+PB; b: TAA+EHEN+B[a]P; c: 2-AAF+3′-Me-DAB+carbazole; d: TAA+EHEN+α-HCH.

of the lesions. This may be partly due to the fact that larger foci might be formed by several small foci coalescing. In the context of the present findings, it is of interest that the area of preneoplastic lesions is generally assumed to reflect promoting activity of chemicals rather than their initiating potential, which bears a close relation to numbers.[32]

In the promoting stage during which altered cells grow to form detectable foci or nodules and finally malignant cell populations, increasing attention has been devoted to possible underlying epigenetic mechanisms. In particular, different susceptibility to cytotoxic effects chemicals between altered and normal cells leading to compensatory regeneration occurring primarily in enzyme-altered foci after partial hepatectomy has been proposed especially for 2-AAF.[13, 33, 34] For the other hepatotoxic agents such as TAA and 3′-Me-DAB, differential toxicity and therefore ability to proliferate might also be directly involved. This effect may have been responsible for the observed synergism between test chemicals.

Important mechanisms underlying this phenomenon may also include altered metabolic conditions in the liver due to enzyme induction or inhibition, leading to increased production of effective chemical metabolites. Although direct evidence for this is limited, the combination of 2-AAF+3′-Me-DAB+PB, which proved an exception in not demonstrating synergism, may be a case in point. The result could have been due to an alteration of the enzymatic conditions in the liver cells: PB is a mixed-function oxidase inducer and the inhibitory effect of simultaneous PB treatment on 2-AAF and 3′-Me-DAB induced hepatocarcinogenesis has been well documented.[14, 15] A similar inhibitory effect might also have been operating in the group in which PB was given with 5 other chemicals; the ratio to the sum total in this later case was only 1.75. Inhibitory effects on hepatocarcinogenesis have also been observed for polychlorinated biphenyls given with 3′-Me-DAB, 2-AAF, and DEN[7] and for α-naphthyl isothiocyanate with ethionine and 2-AAF.[9]

The synergistic effects of the chemicals in this study were much clearer in groups that had not received DEN initiation, this presumably reflecting the fact that the test chemicals at the low doses used were far more effective as promoters than as initiators.

Since most human cancers may be caused by trace environmental factors,[6] it is of increasing importance that combined effects of chemicals at relatively low doses be examined. Knowledge in this area should be of great assistance to public education for cancer prevention.[35] The presented medium-term bioassay system has been shown to be useful not only for detection of tumor-promoting activity but also for examination of agents inhibiting hepatocarcinogenesis.[18] As was clear from the present findings, the system may also be of particular use for medium-term analysis of synergistic and summation effects of environmental chemicals. It should be borne in mind that equivalent long-term experiments would be prohibitively expensive given the large number of animals involved.

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