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Combination Effects of Forty Carcinogens Administered at Low Doses to Male Rats

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An investigation was conducted to determine whether a mixture of low doses of forty carcinogens that target different organs, including the liver, intestine, thyroid, urinary bladder, and skin, is effective for tumor induction in F344/DuCrj rats. The dose of each carcinogen in the diet was 1/50 of the TD_{50} value, treatment being continued for 102 weeks. Significant numbers of neoplastic nodules of the liver and follicular cell tumors of the thyroid developed in the animals exposed to the carcinogen mixture, although the question of whether the observed carcinogenic effects were synergistic or additive could not be answered. The results serve to evaluate carcinogenic risk in the search for causes of human cancer.

Key words: Complex mixtures — Forty carcinogens — Male rat carcinogenesis

Our environment contains a great variety of carcinogenic factors, including naturally occurring or synthetic chemical carcinogens, radiation and viruses, and it has been speculated that they play a role in the etiology of human cancers. Epidemiological studies, for example, have contributed to our understanding of the importance of chemicals in occupational cancer, although clear cases of association are limited. In general populations, there have also been indications that environmental chemicals including aflatoxin B_1, N-nitroso compounds and metals can cause tumor development in man. However, in spite of the numerous carcinogenic agents detected in the environment, there is an unfortunate lack of convincing evidence for specific associations with cancer in particular organs.

Since man is simultaneously exposed to a great variety of environmental carcinogens at only very low individual doses for the lifetime, there is a possibility that the agents may act in combination to induce cancers. In fact, there is an abundance of experimental data showing that different compounds can exert synergistic or additive activity in a wide spectrum of target organs.[1-5] Therefore, examination of low dose combination effects of carcinogens is an important area for research aimed at evaluation of human cancer risk factors.

The present two-year experimental rat study was therefore performed to assess whether combined administration of 40 environmental carcinogens at the doses of corresponding to 1/50 of the TD_{50} values for each compound exerts synergistic or additive effects. Treatment via the diet was selected because oral ingestion is the most probable route of general human exposure.

MATERIALS AND METHODS

Chemicals Forty chemicals, which were reported to be carcinogenic to F344 rats in long-term oral carcinogenicity studies, were selected for the investigation.[6-15] Chemical names, TD_{50} and 1/50 TD_{50} values,[16] calculated dietary levels and target sites are summarized in Table I. Included as target sites were the liver, thyroid, urinary bladder, skin, Zymbal's gland, small intestine, large intestine, hematopoietic organs, etc. Twenty out of the 40 carcinogens chosen exhibited two or more target sites. They were purchased from the following suppliers (carcinogens nos. 2, 3, 6, 12, 15 and 19, Aldrich Chemical Co., USA; no. 23, CIBA Pharmaceutical Co., Switzerland; no. 32, Wako Pure Chemical Industries, Ltd., Osaka; nos. 33, 34 and 37, Nard Institute, Osaka; nos. 35 and 36, Katsura Chemical Co., Tokyo; no. 38, Saber Laboratories, Inc., USA; no. 39, Sigma Chemical Co., USA; and the remainder, Tokyo Kasei Co., Tokyo). Purities of most of the chemicals were more than 99%.

Animals and maintenance Sixty male F344/DuCrj rats were obtained from Charles River Japan, Inc., Kanagawa. The animals were about 6 weeks old at the commencement and were housed five to a wire cage. The room temperature was maintained at 22 ± 2°C and the relative humidity at 60 ± 10% with a 12-h light/dark cycle.

Experimental procedure Thirty male rats were given diet (CE-2, CLEA, Tokyo) containing the mixture of 40 chemical carcinogens for 102 weeks, the remaining animals serving as controls maintained on basal diet. The 1/50 TD_{50} dose value for each chemical used in the investigation was calculated from the results of previously reported carcinogenicity experiments.[6-15] The animals were observed daily for abnormalities; rats found dead or becoming moribund were autopsied. Individual body weights and food intake were recorded monthly.

Gross observation was performed at autopsy, and all major organs or tissues of each of the rats were fixed in 10% buffered formalin. Preserved tissues to be examined microscopically were embedded in paraffin, sectioned,

Table I. Data for the Forty Carcinogens Used in the Experiment

Carcinogens		TD_{50} value reported (mg/kg/day)	1/50 of the TD_{50} value (mg/kg/day)	Dietary concentration applied (ppm)	Target sites
No.	Chemical name				
1	Acetamide	100	2.0	50	liver
2	3-Amino-9-ethylcarbazole HCl	28	0.56	14	liver, skin, Zymbal gland
3	4-Amino-2-nitrophenol	310	6.2	160	urinary bladder
4	2-Amino-5-nitrothiazole	28	0.56	14	hematopoietic organs, multiple organs
5	2-Aminoanthraquinone	100	2.0	50	liver
6	Aniline HCl	88	1.8	44	spleen, multiple organs
7	Anisidine HCl	32	0.64	16	kidney and pelvis, thyroid, urinary bladder
8	Azobenzene	19	0.38	9.5	spleen, liver, multiple organs
9	*p*-Benzoquinone dioxime	300	6.0	150	urinary bladder
10	4-Chloro-*o*-phenylenediamine	200	4.0	100	forestomach, urinary bladder
11	*p*-Chloroaniline	72	1.4	36	spleen
12	Clofibrate	170	3.4	85	liver
13	*p*-Cresidine	76	1.5	38	urinary bladder, liver, nasal cavity
14	Cupferron	5.3	0.11	2.7	forestomach, hematopoietic organs, liver
15	Dapsone	22	0.44	11	spleen
16	2,4-Diaminoanisole sulfate	73	1.5	37	thyroid, Zymbal gland, preputial gland, skin
17	4,4′-Diaminodiphenyl ether	16	0.32	8.0	thyroid, liver
18	2,4-Diaminotoluene	2.5	0.05	1.3	subcutaneous tissue
19	*N,N′*-Diethylthiourea	20	0.48	12	thyroid
20	2,4-Dinitrotoluene	9.4	0.19	4.7	liver, skin, subcutaneous tissue
21	Hydrazobenzene	3.6	0.072	1.8	liver, Zymbal gland
22	Michler's ketone	6.7	0.13	3.4	liver
23	Nafenopin	11	0.22	5.5	liver
24	Nitrilotriacetic acid trisodium salt I	510	10	260	kidney, urinary bladder, urether
25	5-Nitro-*o*-anisidine	28	0.56	14	skin, Zymbal gland
26	5-Nitroacenaphthene	6.5	0.13	3.3	Zymbal gland, lung
27	*N*-Nitrosodiphenylamine	300	6.0	150	urinary bladder
28	Reserpine	0.31	0.0062	0.16	adrenal
29	4,4′-Thiodianiline	5.5	0.11	2.8	thyroid, colon, liver
30	*o*-Toluidine HCl	23	0.46	12	multiple organs, spleen, subcutaneous tissue
31	2,4,6-Trichlorophenol	410	8.2	210	hematopoietic organs
32	Tris(2,3-dibromopropyl)phosphate	1.6	0.032	0.80	kidney
33	3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]-indole	0.11	0.0022	0.055	liver
34	3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole	14	0.28	7.0	liver
35	2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]-imidazole	1.2	0.024	0.60	liver, small and large intestines, Zymbal gland
36	2-Aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole	5.7	0.11	2.9	liver, small and large intestines, Zymbal gland
37	2-Amino-3-methylimidazo[4,5-*f*]quinoline	0.33	0.0066	0.17	Zymbal gland, colon, small intestine, liver, skin
38	*N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide	1.7	0.034	0.85	urinary bladder
39	2-Acetylaminofluorene	0.023	0.00046	0.011	liver
40	3′-Methyl-4-dimethylaminoazobenzene	0.097	0.0019	0.049	liver

and stained with hematoxylin and eosin. A full histopathological examination was performed on all rats sacrificed at the termination of the experiment, and also on these that died or were killed on becoming moribund.

Statistical analysis Data on cumulative mortality and tumor incidence were analyzed by the two-sided Fisher's exact probability test, and Student's *t*-test for statistical analysis of the differences in average survival times and average times of detection of tumors.

RESULTS

No remarkable clinical signs or mortality were evident in the carcinogen-treated rats as compared to the controls. Slight retardation of body weight increase was apparent in the rats treated with the carcinogen mixture from around 6 months until the end of the experiment. No marked difference in food consumption was noted between control and carcinogen-treated rats.

Table II. Preneoplastic and Neoplastic Lesions Developing in Organs, Targeted by Two or More Carcinogens, of F344 Rats Receiving the Mixture of 40 Carcinogens in the Diet

Site and type of lesion	Control	40 carcinogens
Effective no. of rats	30	29
Spleen		
Histiocytoma	0	1 (3)
Hemangioendothelioma	0	1 (3)
Thyroid		
Follicular cell tumor	0	5 (17)*
Follicular cell adenoma	0	2 (7)
Follicular cell carcinoma	0	3 (10)
C-cell tumor	5 (17)	5 (17)
C-cell adenoma	4 (13)	5 (17)
C-cell carcinoma	1 (3)	0
Small intestine		
Leiomyosarcoma	1 (3)	0
Liver		
Focus of cellular alteration	11 (37)	7 (24)
Neoplastic nodule	3 (10)	17 (59)**
Hepatocellular carcinoma	0	1 (3)
Kidney		
Transitional cell carcinoma	0	1 (3)
Urinary bladder		
Transitional cell papilloma	1 (3)	1 (3)
Abdominal cavity		
Mesothelioma	1 (3)	1 (3)
Whole body		
Malignant lymphoma/leukemia	2 (7)	0

*, ** Significantly different from control values at $P<0.05$ and 0.01, respectively.

Incidences of preneoplastic and neoplastic lesions which developed in organs treated with the forty carcinogens are summarized in Table II. Histopathological examination of the liver revealed a markedly increased incidence of neoplastic nodules[17] in carcinogen-treated rats. However, no difference in the incidences of foci of cellular alteration was found between the control and treated groups. One hepatocellular carcinoma was observed in a rat receiving the mixture of 40 carcinogens.

Two follicular cell adenomas and three follicular cell carcinomas of the thyroid were found in the treated group, no such lesions being obseved in the controls; the incidence (follicular cell adenomas plus follicular cell carcinomas) is statistically significant. No difference in development of C-cell tumors was apparent.

In the other target organs, treatment-related changes in the tumor incidence were not observed. Only one urinary bladder transitional cell tumor was found in the treated group in spite of exposure to 8 urinary bladder carcinogens, and a papilloma also developed spontaneously in the controls.

No treatment-related tumor development or modification of the incidences of spontaneous tumors was apparent. However, enhanced severity of chronic nephropathy[18] with renal tubular calcification was apparent in rats of the treated group. The other non-neoplastic lesions observed were equivalent to those considered normal in aged F344 rats.

DISCUSSION

The results of the present experiment demonstrated that combined administration of 40 carcinogens at very low doses for 2 years induced neoplastic nodules of the liver and follicular cell tumors of the thyroid in male F344 rats. Although the carcinogenic potential of each individual liver and thyroid carcinogen alone at doses of 1/50 TD_{50} was not examined in the present investigation, very little or no tumorigenic response in these target organs would be expected. Therefore, combination effects, either additive or synergistic, for the liver and thyroid carcinogens employed were suggested to have occurred.

In general, synergism of carcinogenic response has been defined as occurring when the effect of two or more substances acting together exceeds the sum of their effects when acting separately.[19] There are many experimental data[4, 5, 20–22] demonstrating that various carcinogens can act synergistically on their target organs. However, these studies were performed at relatively high doses, with tumors also being induced by the single chemicals. In liver carcinogenesis, for example, it was demonstrated that combination treatment with N-nitroso compounds exhibits a synergistic effect in rats.[23] Re-

cently, a large-scale carcinogen mixture experiment reported a similar enhancement of response to liver carcinogens in pairwise combinations.[3, 20] We also detected combined effects of 5 heterocyclic amines, which are known to be present in cooked food, in liver carcinogenesis of rats.[24] In addition, a simple additive influence, equal to the sum of the effects of two or more carcinogens, has been observed.[3, 19] The results of the present study therefore reflect either an additive or a synergistic influence of the hepatocarcinogens employed, although studies on the carcinogenic potential of each of the agents applied at 1/50 TD_{50} are required for firm conclusions regarding this question to be drawn.

There is no report concerning organotropic combination effects of thyroid carcinogens at low doses. The fact that several thyroid carcinogens at dose levels of 1/50 TD_{50} exerted combination effects for thyroid carcinogenesis in the present study is therefore of interest.

Combination effects of environmental carcinogens for the urinary bladder, kidney, skin, small and large intestines were not obtained in the present study. This might indicate that the combined levels of carcinogens active in these organs were below the threshold for tumor induction within two years, since a no-effect level is thought to exist for carcinogens in combination.[23] A study on the presence of the added carcinogens in the diet one year after preparation is in progress. However, the situation is complicated by the possibility of antagonistic interaction between the mixture of carcinogens given in the diet. Further large-scale studies are therefore required to elucidate the mechanisms involved in combined carcinogenicity in different target organs.

In conclusion, the problem of combination effects of carcinogens at low doses is very complex. Nevertheless, this research area is obviously of great importance for evaluation of carcinogenic risk factors for man. The fact that we cannot escape ingestion of many environmental carcinogens at very low levels, such as in cooked foods,[25] dictates that further assessment of this question be made.

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REFERENCES

1) Ito, N., Hiasa, Y., Konishi, Y. and Muragami, M. The development of carcinoma in liver of rats treated with *m*-toluenediamine and the synergistic and antagonistic effects with other chemicals. *Cancer Res.*, **29**, 1137–1145 (1969).
2) Lijinski, W., Reuber, M. D. and Riggs, C. W. Carcinogenesis by combinations of N-nitroso compounds in rats. *Food Chem. Toxicol.*, **21**, 601–605 (1983).
3) Arcos, J. C., Woo, Y. T. and Lai, D. Y. Database on binary combination effects of chemical carcinogens. *Environ. Carcinog. Rev.*, **6**, 1–150 (1988).
4) Shellabarger, C. J., Machado, S. G., Holtzman, S. and Stone, J. P. Assessment of interaction among three carcinogens on rat mammary carcinogenesis in a factorially designed experiment. *J. Natl. Cancer Inst.*, **79**, 549–554 (1987).
5) Tsuda, H., Miyata, Y., Murasaki, G., Kinoshita, H., Fukushima, S. and Ito, N. Synergistic effect of urinary bladder carcinogenesis in rats treated with N-butyl-N-(4-hydroxybutyl)nitrosamine, N-[4-(5-nitro-2-furyl)2-thiazolyl]formamide, N-2-fluorenylacetamide, and 3,3′-dichlorobenzidine. *Gann*, **68**, 183–192 (1977).
6) Gold, L. S., Sawyer, C. B., Magaw, R., Backman, G. M., de Veciana, M., Levinson, R., Hooper, N. K., Havender, W. R., Bernstein, L., Peto, R., Pike, M. C. and Ames, B. N. A carcinogenic potency database of the standardized results of animal bioassays. *Environ. Health Perspect.*, **58**, 9–319 (1984).
7) Hosaka, S., Matsushima, T., Hirono, I. and Sugimura, T. Carcinogenic activity of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), a pyrolysis product of tryptophan. *Cancer Lett.*, **13**, 23–28 (1981).
8) IARC Monographs on the evaluation of carcinogenic risks to humans, Vol. 29. "Some Industrial Chemicals and Dyestuffs, *para*-Benzoquinone Dioxime," pp. 185–191 (1982). International Agency for Research on Cancer, Lyon.
9) IARC Monographs on the evaluation of carcinogenic risks to humans, Vol. 24. "Some Pharmaceutical Drugs, Nafenopin," pp. 125–133 (1982). International Agency for Research on Cancer, Lyon.
10) National Cancer Institute. Bioassay of 4,4′-oxydianiline for possible carcinogenicity (DHEW Pub. No. (NIH) 80-1761). *Tech. Rep. Ser.*, **n205** (1980). US Department of Health, Education and Welfare, Washington, DC.
11) Smith, C. J. and Kelleher, P. C. Induction of hepatomas secreting large amounts of alpha-fetoprotein. *Cancer Lett.*, **3**, 53–57 (1977).
12) 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).

13) 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).
14) 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.*, **76**, 815–817 (1985).
15) Yamamoto, R. S., Glass, R. M., Frankel, H. H., Weisburger, E. K. and Weisburger, J. H. Inhibition of the toxicity and carcinogenicity of N-2-fluorenylacetamide by acetanilide. *Toxicol. Appl. Pharmacol.*, **13**, 108–117 (1968).
16) Peto, R., Pike, M. C., Bernstein, L., Gold, L. S. and Ames, B. N. The TD_{50}: a proposed general convention for the numerical description of the carcinogenic potency of chemicals in chronic-exposure animal experiments. *Environ. Health Perspect.*, **58**, 1–8 (1984).
17) Institute of Laboratory Animal Resources. Histologic typing of liver tumors of the rat. *J. Natl. Cancer Inst.*, **64**, 179–206 (1980).
18) Hayashi, Y., Takahashi, M. and Kokubo, T. Nutritional factors influencing the results of toxicology experiments in animals. *J. Toxicol. Sci.*, **9**, 219–234 (1984).
19) Reif, A. E. Synergism in carcinogenesis. *J. Natl. Cancer Inst.*, **73**, 25–39 (1984).
20) Elashoff, R. M., Fears, T. R. and Schneiderman, M. A. Statistical analysis of a carcinogen mixture experiment. I. Liver carcinogens. *J. Natl. Cancer Inst.*, **79**, 509–526 (1987).
21) Harrison, P. T. C. and Heath, J. C. Apparent synergy in lung carcinogenesis: interactions between N-nitrosoheptamethyleneimine, particulate cadmium and crocidolite asbestos fibres in rats. *Carcinogenesis*, **7**, 1903–1908 (1986).
22) Peraino, C., Grdina, D. J. and Carnes, B. A. Synergistic induction of altered hepatocyte foci by combined gamma radiation and diethylnitrosamine administered to neonatal rats. *Carcinogenesis*, **7**, 445–448 (1986).
23) Berger, M. R., Schmähl, D. and Zerban, H. Combination experiments with very low doses of three genotoxic N-nitrosamines with similar organotropic carcinogenicity in rats. *Carcinogenesis*, **8**, 1635–1643 (1987).
24) Takayama, S., Nakatsuru, Y. and Sato, S. Carcinogenic effect of the simultaneous administration of five heterocyclic amines to F344 rats. *Jpn. J. Cancer Res.*, **78**, 1068–1072 (1987).
25) Sugimura, T. Successful use of short-term tests for academic purposes: their use in identification of new environmental carcinogens with possible risk for humans. *Mutat. Res.*, **205**, 33–39 (1988).

Note added in proof: After submission of the manuscript, we have learned that Michael D. Shelby *et al.* reported "Toxicity and mutagenicity of mixture of 25 chemicals found in contaminated fround water" in the Proceedings of the Workshop on Experimental and Epidemiologic Applications to Risk Assessment of Complex Mixtures, Helsinki, Finland, May 14–17, 1989.