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Effects of ingesting a combination of 20 or 40 pesticides at ADI levels on carcinogenesis in rats

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Abstract. Modifying effects of pesticide mixtures on tumorigenesis were investigated with our medium-term carcinogenesis protocols. In the 8-week rat liver model, administration of 20 pesticides (19 organophosphorus and one organochlorine), added to the diet each at acceptable daily intake (ADI) levels, did not enhance rat liver preneoplastic lesion development initiated by diethylnitrosamine. In contrast, a mixture of these 20 pesticides at 100 times the ADI significantly increased the number and area of liver lesions. In a multi-organ carcino-genicity protocol of 28 weeks, mixtures of 40 pesticides (high production examples) or 20 pesticides (suspected carcinogens) added to the diet at their respective ADI levels did not modulate carcinogenesis in any organ initiated by 5 carcinogens in combination. These results thus provide direct support for the safety factor approach using ADI values for the quantitative risk valuation of pesticides. Furthermore, these bioassays were particularly useful methods for the safety evaluation of combination toxicities.

1. Introduction

As possible environmental toxic or carcinogenic agents, pesticides deserve particular attention [3,15]. Not only are workers in the industrial and agricultural fields exposed to these chemicals, but the general public is also potentially at risk due to exposure to pesticide residues in foods. Although the assessment of human cancer risk associated with specific chemical exposures is a complicated scientific endeavor, the WHO Expert Group on Pesticide Residues and the Food and Agriculture Organization of the United Nations (FAO), which regularly hold joint meetings on pesticide residues, have set an acceptable daily intake (ADI) for each pesticide as one approach to quantitative risk evaluation [4]. Actually, pesticide residues were found in many samples and some (about 1 % of 3300 chemicals) exceeded maximum residue limits [2].

We have conducted an extensive study of the carcinogenic activity of pesticides in the last few years using our medium-term bioassay system [1,5,9]. In order to confirm the efficacy of this approach using ADIs, and since additive or synergistic

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effects of complex mixtures have become increasingly important for risk estimation in human toxicity [11,14,17,19], we tested the possible carcinogenic influence of mixtures of 20 or 40 pesticides. To do this we used two types of medium-term bioassays for rapid detection of carcinogens; the liver and multi-organ models [11]. All pesticides examined are registered for use in Japan at the present time.

2. Materials and methods

Male F344 rats, 6 weeks old, were used. The ADI values were proposed by the Ministry of Health and Welfare, Japan, according to the JMPR reports [4]. Concentrations of pesticides in the diet (mg/kg diet) were calculated based on our previous food intake and body weight data [10]. Food and water were available *ad libitum*.

Experiment 1: The pesticides investigated, along with concentrations in the diet, their purity and ADIs (mg/kg body weight/day) were: acephate, 0.3 mg/kg (99.3 %, 0.03); butamifos, 0.016 mg/kg (97.9 %, 0.0016); chlorfenvinphos, 0.015 mg/kg (93.3 %, 0.0015); chlorpyrifos, 0.1 mg/kg (99.3 %, 0.01); dichlorvos, 0.033 mg/kg (98.9 %, 0.0033); dimethoate, 0.1 mg/kg (99.0 %, 0.01); edifenphos, 0.025 mg/kg (95.0 %, 0.0025); endosulfan, 0.06 mg/kg (98.0 %, 0.006); etrimfos, 0.03 mg/kg (94.0 %, 0.003); fenitrothion, 0.05 mg/kg (96.7 %, 0.005); iprobenfos, 0.03 mg/kg (94.9 %, 0.003); isoxathion, 0.03 mg/kg (95.2 %, 0.003); malathion, 0.2 mg/kg (95.4 %, 0.02); methidathion, 0.01 mg/kg (92.04 %, 0.001); pirimiphos-methyl, 0.1 mg/kg (99.7 %, 0.01); prothiophos, 0.015 mg/kg (94.7 %, 0.0015); pyraclofos, 0.01 mg/kg (98.4 %, 0.001); tolclofos-methyl, 0.64 mg/kg (99.5 %, 0.064); trichlorfon, 0.1 mg/kg (99.0 %, 0.01); and vamidothion 0.08 mg/kg (99.0 %, 0.008). All pesticides examined are organophosphorus compounds, except for endosulfan.

Fig. 1 shows the experimental protocol for Experiment 1. The animals were initially given a single i.p. injection of diethylnitrosamine (DEN) at a dose of 200 mg/kg. After a 2-week recovery period, the rats received the pesticides either at the ADI level (mixture 1, group 1-a) or at 100 times ADI (group 1-b), or were maintained on the basal diet throughout the experiment (group 2). Groups 3-a and 3-b were injected with saline and then fed on mixture 1 at the ADI and 100 times ADI, respectively. All animals were subjected to two-thirds partial hepatectomy at week 3 and sacrificed at week 8. Liver slices were fixed in ice-cold acetone, embedded in paraffin, and then immunohistochemically stained for glutathione S-transferase placental form (GST-P) – as previously reported [1]. Numbers and areas of GST-P-positive hepatic cell foci larger than 0.2 mm in diameter, and the total area of liver sections examined, were measured using a video image processor.

Experiment 2: Pesticides selected for the mixtures were 40 chemicals of high volume production (mixture 2) and 20 chemicals for which carcinogenicity has been reported or suspected (mixture 3). The pesticides and concentrations (mg/kg diet) were: acephate (0.3), bendicarb (0.04), bensulide (0.4), bentazone (0.9), chinomethionat (0.06), chlorobenzilate (0.2), chlorpropham (1), chlorpyrifos (0.1),

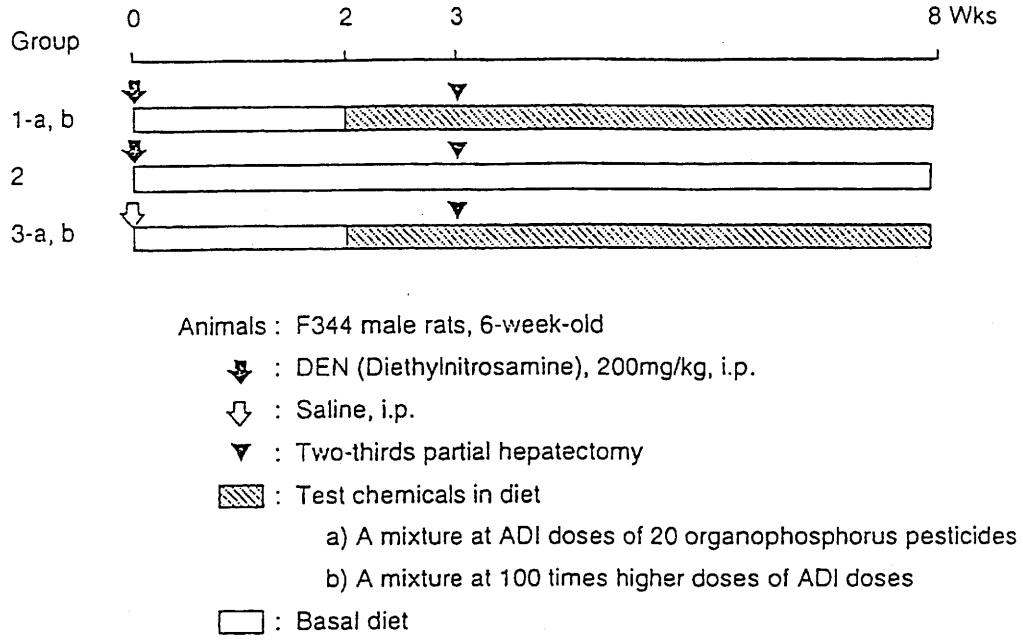

Fig. 1. Experimental protocol of the medium-term liver bioassay (*Experiment 1*)

clofentezine (0.086), cyfluthrin (0.2), cyhalothrin (0.085), cypermethrin (0.5), diflubenzuron (0.12), fenarimol (0.1), fenbutantin oxide (0.3), fenvalerate (0.2), flucythrinate (0.125), flutolanil (0.8), glyphosate (1.5), imazalil (0.25), malathion (0.2), maneb (0.05), mepiquat chloride (0.75), metalaxyl (0.19), metolachlor (0.97), metribuzin (0.125), myclobutanil (0.12), oxamyl (0.2), pendimethalin (0.43), permethrin (0.48), pirimiphos-methyl (0.1), propiconazole (0.18), pyrifenox (1), quinclorac (0.29), methoxydim (1.4), thiobencarb (0.09), triadimefon (0.12), trichlorfon (0.1), vinclozolin (1.215), and zineb (0.05) in mixture 2, and acephate (0.3), amitraz (0.012), captafol (0.5), clofentezine (0.086), cypermethrin (0.5), 2,4-D (3), dichlorvos (0.033), dichlobenil (0.04), dicofol (0.25), fosetly (8.8), glyphosate (1.5), mancozeb (0.5), maneb (0.05), mefolachlor (0.97), permethrin (0.48), phosmet (0.2), propiconazole (0.18), propoxur (0.63), triadimefon (0.12), and trifluralin (0.075) in mixture 3.

Possible modifying effects of these pesticide mixtures on tumorigenesis were investigated using the medium-term multi-organ bioassay (DMBDD model, Fig. 2) [11,6]. At initiation, five known potent carcinogens were given in combination within the first 4 weeks; a single i.p. injection of DEN at a dose of 100 mg/kg body weight at the start of the experiment, i.p. injections of N-methyl-N-nitrosourea (MNU) at a dose of 20 mg/kg body weight on days 2, 5, 8, and 11, and 4 s.c. injections of 1,2-dimethylhydrazine (DMH) at a dose of 40 mg/kg body weight on days 14, 17, 20, and 23, 500 mg/liter N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) in the drinking water during weeks 1 and 2, and 1000 mg/liter 2,2'-dihydroxy-di-n-propylnitrosamine (DHPN) in the drinking water during weeks 3 and 4.

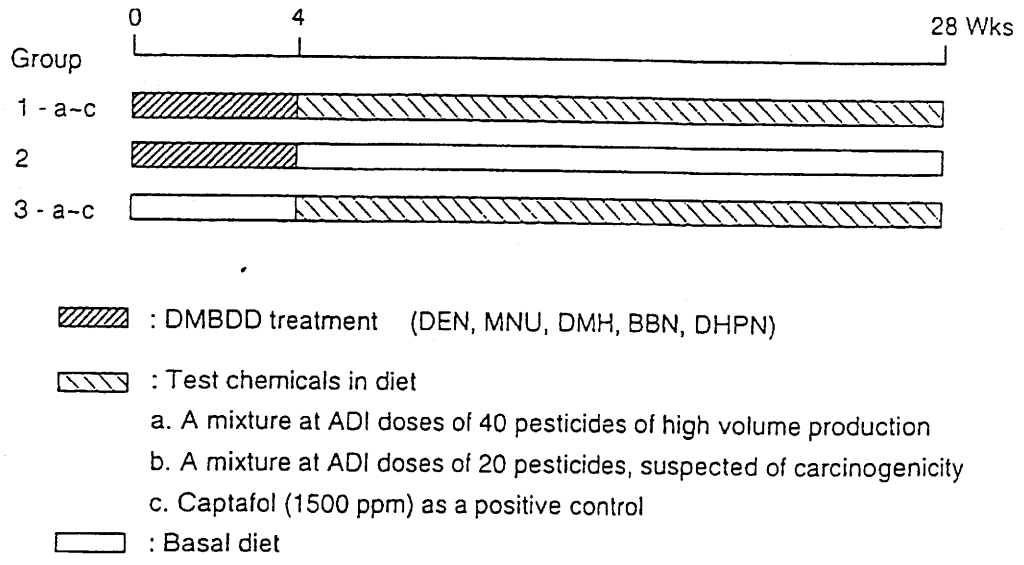

Fig. 2. Experimental protocol of the multi-organ bioassay (*Experiment 2*)

After this DMBDD treatment, groups of rats received one of the pesticide mixtures (group 1 for mixture 2 and group 2 for mixture 3), captafol (1500 mg/kg in the diet) as a positive control (group 3) [16], or the basal diet (group 4) for 24 weeks. Non-initiation controls were injected i.p. with saline and subcutaneously with corn oil and then given pesticide(s) (groups 5–7). At week 28 of the experiment, all surviving animals were killed and completely autopsied. Livers were analyzed as in Experiment 1. The small and large intestines, lungs, and urinary bladders were inflated with 10 % phosphate – buffered formalin, and other main organs and any macroscopic lesions were removed and fixed in formalin. The routinely prepared hematoxylin and eosin sections were examined for neoplastic and preneoplastic lesions.

3. Results

Experiment 1: Based on the food consumption values and average body weights, actual chemical intake was found to be slightly lower than the estimated intake. Body and liver weights were also not influenced by the pesticide administration.

Data on the numbers and areas of GST-P-positive foci per unit area of liver section with and without DEN-initiation are illustrated in Fig. 3. The number of GST-P-positive foci in group 1-a was $3.36 \pm 1.29/cm^2$ and the area of foci was $0.29 \pm 0.15\ mm^2/cm^2$. The levels were essentially the same as those found in the control group ($3.50 \pm 1.29/cm^2$ and $0.28 \pm 0.13\ mm^2/cm^2$). However, the values obtained in the 100 times ADI mixture group (group 1-b) ($4.51 \pm 1.64/cm^2$ and $0.44 \pm 0.20\ mm^2/cm^2$) were both significantly higher than those found in the controls. Without the DEN initiation, neither of the treatment schedules induced GST-P-positive liver cell foci larger than 0.2 mm in diameter (groups 3-a and 3-b).

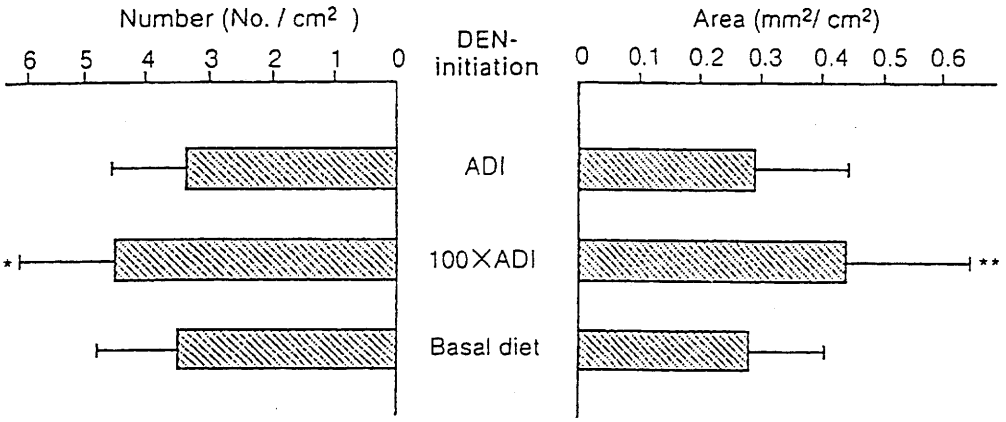

Fig. 3. Numbers and areas of GST-O-positive liever cell foci in *Experiment 1*.
**,* significantly different from basal diet group at $P < 0.01$, $P < 0.05$, respectively.

Experiment 2: No toxic effects were observed for pesticide mixtures in terms of survival rates and weight data. In the liver, development of GST-P-positive foci was increased by captafol but not modulated by the mixtures (Fig. 3). In the other organs, captafol showed promotion effects in the thyroid, whereas the pesticide mixtures did not influence the neoplastic development in any organ (Table 1). No neoplastic and preneoplastic lesions were observed in non-initiated groups (groups 3-ac).

4. Discussion

In the liver model, the ADI mixture of organophosphorus pesticides (mixture 1) exerted no effect on development of liver preneoplastic foci initiated by DEN, although

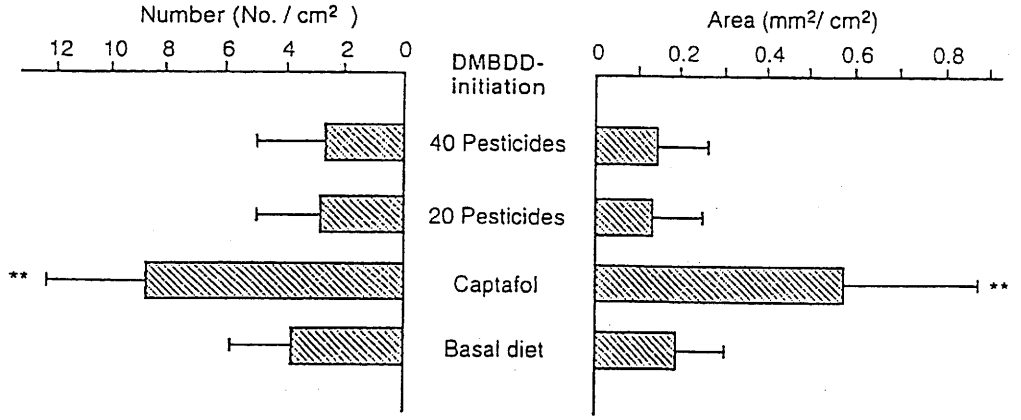

Fig. 4. Numbers and areas of GST-P-positive lever cell foci in *Experiment 2*.
** significantly different from basal diet group at $P < 0.01$.

Table 1. Incidence of tumours (Experiment 2)

Organ and type of tumours	DMBDD-treated group			
	40 pesticides	20 pesticides	Captafol	Basal diet
No. of animals	20	20	19	20
Thyroid				
Follicular adenoma	2	6	9*	2
C-cell adenoma				
Nasal cavity				
Papilloma	1	0	0	0
Odontoma	0	1	0	0
Lung				
Adenoma	4	5	3	5
Carcinoma	1	1	0	2
Oral cavity				
Odontoma	0	2	0	0
Esophagus				
Squamous cell carcinoma	0	0	1	0
Forestomach				
Squamous cell papilloma	3	8	2	4
Squamous cell carcinoma	0	1	1	0
Small intestines				
Adenoma	3	1	2	2
Adenocarcinoma	0	2	2	1
Large intestines				
Leiomyoma	0	1	0	0
Adenocarcinoma	2	2	6	4
Liver				
Hyperplastic nodule	1	0	1	1
Kidney				
Nephroblastoma	2	4	7	2
Transitional cell carcinoma	0	1	0	0
Urinary bladder				
Transitional cell papilloma	0	1	0	1
Prostate				
Leiomyosarcoma	0	1	0	0
Sarcoma, NOS	1	1	0	0
Skin/subcutis				
Squamous cell papilloma	0	1	0	0
Lipoma	0	1	0	0
Abdominal cavity				
Mesothelioma	0	0	1	0
Peripheral nerve				
Malignant schwannoma	0	1	0	0

*: Significantly different from control group at $P < 0.05$.

A few tumors were observed only in the control group: thymic lymphoma (thymus), follicular carcinoma (thyroid), adenocarcinoma (nasal cavity), adenoma (seminal vesicle), keratoacanthoma (skin), schwannoma (peripheral nerve). No neoplastic lesions were found in the non.initiated groups.

the 100 times higher dose demonstrated lesion-promoting potential [10,8,7]. In the multi-organ model, the ADI mixtures of 40 (mixture 2) or 20 (mixture 3) pesticides demonstrated no tumor-promoting potential in any organ or tissue [8,7]. Captafol, on the other hand, exerted apparent tumor- promoting effects in the liver, thyroid and kidney, although the dose level was not comparable to the mixtures. The protocol has been developed in our laboratory over the last 15 years [11]. Quantitative analysis of GST-P-positive foci larger than 0.2 mm in diameter, expressed in terms of number and area per unit area of liver section, has been established. The multi-organ method has been developed to supplement the liver model and also has been demonstrated to be a useful method for rapid detection of carcinogens at a whole body level [11,6].

With a safety factor approach, acceptable exposure levels such as ADIs are usually determined by dividing the no observed effect level (NOEL) from laboratory-based chronic toxicity tests by an appropriately chosen safety factor. The safety factor used for ADI by the Japanese Ministry of Health and Welfare and the FAO/WHO is usually 100, but the WHO expert committees have used figures ranging from 10 to 2000 [13]. Although there are a number of potential problems associated with the safety factor approach, including the fact that the observation of no treatment-related effects may depend on experimental conditions (such as the number of animals exposed and dose levels used), and the fact that biological justification for general use may be lacking [12], the present experimental results indicate that this procedure is indeed appropriate and acceptable for risk evaluation at present. Furthermore, the chance of exposure to so many pesticides (20 or 40 chemicals) in concert might be in practice very low [18].

The observed combination effects at 100 times ADI in Experiment 1, however, suggest that several of the pesticides included in the test are possibly carcinogenic in the liver. Even the mixture of 20 pesticides at ADI levels , for which carcinogenicity has been reported or suspected, exerted no tumor modulating potential in the DMB-DD model.

Since most human cancers may be caused by trace environmental factors, it is of increasing importance that combined effects of chemicals at relatively low doses be examined. The medium-term bioassays used in this document are particularly useful methods for this matter. In conclusion, the present safety factor approach is appropriate for risk evaluation of environmental chemicals.

Acknowledgements

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