Table 1. Chloroform vapor concentration at each sampling port

Parts name	Sampling port	Study-1 [a] Mean ± S.D. (ppm)	Study-1 C.V. (%)	Study-2 [b] Mean ± S.D. (ppm)	Study-2 C.V. (%)
Supply-header	S-1	101.5 ± 0.83 [c]	0.82	101.1 ± 1.87	1.85
	S-2	100.7 ± 0.77	0.77	100.9 ± 1.53	1.52
Animal-chamber	A-1	100.2 ± 1.32	1.32	100.8 ± 0.61	0.61
	A-2	100.8 ± 0.84	0.83	101.3 ± 1.50	1.48
	A-3	101.1 ± 1.43	1.41	100.5 ± 1.16	1.16
	A-4	100.6 ± 1.90	1.87	100.7 ± 1.27	1.26
	A-5	100.0 ± 1.58	1.58	100.9 ± 0.98	0.97
	A-6	101.2 ± 1.89	1.87	100.9 ± 1.22	1.21
Exhaust-header	E-1	100.6 ± 1.63	1.62	100.1 ± 1.67	1.66

[a] A performance study of the system without rats.

[b] Confirmation of the performance of the system with rats.

[c] The chloroform vapor concentration at each sampling port is expressed as the mean ± S.D. of concentrations measured at every 15 min over the course of a 6 hr exposure period.

er over the course of a 6 hr exposure period were in the range of 100.1 to 101.3 ppm with C.V. being less than 1.85%. The flow rate through each animal-chamber during this period was maintained within a range of 250 to 260 ml/min. Thus, the supply-header was able to supply uniform stable chloroform vapor to each animal-chamber when the chambers contained test animals. In addition, there was no detectable chloroform vapor at the rat tail outlet of the animal-chambers, indicating that the backup-plate and silicon-septum formed an effective seal against the tail root of the rat. Overall, the performance of the system was rated as excellent.

Use the system to analyze the concentration chloroform in the blood of test animals during the exposure to chloroform vapor (study 3)

Rats were exposed to chloroform vapor for 6 hr, and blood was collected from each animal during the course of the exposure period. The chloroform vapor concentration (mean ± S.D.) at the sampling ports of the supply-header (S-1 and S-2 in Fig. 1) and exhaust-header (E-1 in Fig.1) was 100.8 ± 0.82, 101.6 ± 0.81 and 100.8 ± 0.94 ppm, respectively, with C.V. of 0.82 (S-1 in Fig. 1), 0.80 (S-2 in Fig. 1) and 0.93% (E-1 in Fig. 1). The flow rate through each animal-chamber was maintained within a range of 250 to 260 ml/min.

During the inhalation exposure period, blood samples were collected 8 times from each rat. Table 2 shows the chloroform concentration in the blood samples, analyzed by HS-GC/MS. Chloroform was detected in the blood of all rats at 1 min after the start of exposure demonstrating that chloroform was quickly absorbed into the blood stream. The chloroform concentration in blood of all rats showed an increase from 1 to 60 min, and afterwards, its concentration remained in the range from 1.89 to 2.04 μg/ml, with C.V. in the range of 5.02 to 12.96%. The chloroform concentrations in the blood of the different rats was found to very similar. Time-course changes in blood chloroform concentration were also very similar among the different animals.

Table 2. Chloroform concentration in blood samples

Blood collection time (min)	Chloroform concentration in blood Mean ± S.D. (μg/ml)	C.V. (%)
1	0.42 ± 0.05 [a]	12.21
30	1.44 ± 0.08	5.61
60	1.91 ± 0.10	5.02
120	2.04 ± 0.26	12.96
180	1.89 ± 0.20	10.41
240	1.91 ± 0.20	10.60
300	1.98 ± 0.24	11.95
360	1.95 ± 0.25	12.55

[a] n = 6

Finally, six rats were exposed by inhalation to 100 ppm (v/v) of chloroform vapor for 6 hr/day and 5 days/week using the whole-body inhalation exposure system. No marked body weight depression occurred nor were abnormal clinical signs observed in the rats during or after the inhalation exposure period. The system performed within the parameters observed during the single-day exposure period.

In conclusion, blood collection from individual animals was possible during whole-body inhalation exposure to a model VOC. The system should be effective for examining the time-course changes in blood concentra-

tions of other VOCs during exposure. This information can be used as basic data for risk assessment of human health exposure to VOC vapor by inhalation.

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Oral Carcinogenicity and Toxicity of 2-Amino-4-chlorophenol in Rats

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Abstract: Oral Carcinogenicity and Toxicity of 2-Amino-4-chlorophenol in Rats: Kazunori YAMAZAKI, *et al.* Japan Bioassay Research Center, Japan Industrial Safety and Health Association—Objectives: This study was carried out to clarify the subchronic and chronic toxicity, and carcinogenicity of 2-amino-4-chlorophenol(ACP). **Methods:** Carcinogenicity, and chronic and subchronic toxicity of ACP were examined by feeding 10 rats of both sexes ACP-containing diet at a dose level of 0 (control), 512, 1,280, 3,200, 8,000 or 20,000 ppm (w/w) for 13 wk and 50 rats of both sexes at a dose level of 0, 1,280, 3,200 or 8,000 ppm for 2 yr. **Results:** The 13-wk oral subchronic toxicity of ACP was characterized by proliferative lesions leading to development of tumors in the forestomach and urinary bladder and by erythrocyte toxicity as evidenced by decreases in red blood cell counts, hemoglobin and hematocrit and concurrent increases in methemoglobin levels and reticulocyte counts. Both simple and papillary and/or nodular types of transitional cell hyperplasias were observed in the urinary bladder of ACP-fed male rats. The proliferative lesions appeared at higher doses of ACP after the 13-wk administration than clear erythrocyte toxicity did. The 2-yr oral administration of ACP significantly increased incidences of squamous cell papillomas and carcinomas in the forestomach of male and female rats and transitional cell carcinomas in the urinary bladder of male rats. These tumor incidences increased dose-dependently. Notably, clear signs of erythrocyte toxicity were not evident after the 2-yr administration of ACP. **Conclusion:** Clear evidence of carcinogenic activity of ACP was shown in male and female rats. These data might be useful for the health risk assessment of workers exposed to ACP. *(J Occup Health 2009; 51: 249–260)*

Key words: 2-Amino-4-chlorophenol, Erythrocyte toxicity, Forestomach tumor, Hyperplasia, Rat, Urinary bladder tumor

2-Amino-4-chlorophenol (ACP) is used as an intermediate in the chemical and pharmaceutical industries, and as a raw material in dye manufacture[1, 2]. The annual production of ACP in Japan was reported to be 500 tons in 2006[1]. For industrial production of ACP, 2,5-dichloronitrobenzene is converted to 4-chloro-2-nitrophenol by reaction with sodium hydroxide, followed by reduction with iron, hydrazine or hydrogen in the presence of Raney nickel or platinum catalysts[3]. Excessive exposure of workers to ACP in the manufacturing process is unlikely to occur, because ACP is produced in a closed system. However, the possibility that workers might be exposed to ACP during other processes, including packing of synthesized ACP or cleaning or repairing of the ACP-synthesizing reactor, cannot be ruled out.

No epidemiological study on the health effects of occupational exposure to ACP has been reported[2]. ACP is reported to possess a stimulative property that causes irritation and contact dermatitis[4–7]. Two medical examination studies showed contact hypersensitivity to ACP in the patch test among workers handling ACP[6] and a pronounced increase in hemoglobins X and Y and methemoglobin in the erythrocytes of production workers handling aromatic compounds including ACP[8]. Experimental toxicology studies on ACP are very few[2], but it has been reported that bacterial mutagenicity[9, 10] and mammalian chromosome abnormality[11] of ACP are positive with or without S9 activation. ACP has been evaluated as one of the existing chemical substances with

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positive mutagenicity, according to the Labour Standards Bureau Notification in Japan, which requires that occupational health countermeasures be taken to protect workers from exposure to mutagenic substances[12].

No bioassay study of rodent carcinogenicity and chronic toxicity for ACP has been reported[2]. Carcinogenic potential and classification of ACP have not been evaluated by the International Agency for Research on Cancer (IARC), the Japan Society for Occupational Health (JSOH), the American Conference of Governmental Industrial Hygienists (ACGIH) or the German Research Foundation (DFG). A 2-yr bioassay study of rodent carcinogenicity is urgently needed for evaluation of the carcinogenic potential of ACP, since ACP was reported to show positive bacterial mutagenicity and structural and numerical chromosome aberrations[9–11].

The present studies consisted of both a subchronic toxicity study and a carcinogenic and chronic toxicity study, feeding rats of both sexes an ACP-containing diet for 13 wk and 2 yr, respectively. The 13-wk study was undertaken to characterize subchronic toxicity, including possible precursor lesions which might lead to development of neoplasms, and to determine an appropriate range of ACP dose levels for the 2-yr carcinogenicity study. The 2-yr study was undertaken to characterize the dose-response relationships of rat carcinogenicity and chronic toxicity induced by 2-yr oral administration of ACP in feed. The present data for dose-response relationships for two different types of tumors obtained with rats are discussed for their validity in extrapolations to lower dose levels of environmental relevance and to human cancers, for use in risk assessment.

Materials and Methods

The present studies were conducted with reference to two OECD Guidelines: Testing of Chemicals 408 "Subchronic oral toxicity-Rodent: 90-day study"[13] and for Testing of Chemicals 451 "Carcinogenicity Studies"[14]. These studies were approved by the ethics committee of the Japan Bioassay Research Center (JBRC), and performed in conformity with the OECD Principles of Good Laboratory Practice[15].

Test substance

ACP of guaranteed grade (>99.1% pure) was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). The ACP was analyzed for purity and stability by gas chromatography before and after its use. The GC analysis indicated that neither impurities nor degradation products were detected in the test substance.

Animals and husbandry

The animals were cared for in accordance with a guide for the care and use of laboratory animals[16]. Four-week-old F344/DuCrlCrlj rats (SPF) of both sexes were purchased from Charles River Japan, Inc (Kanagawa, Japan). After 2-wk quarantine and acclimation, the animals were allocated by a stratified randomization procedure into body weight-matched, ACP-fed and control groups. The 13-wk study consisted of 5 dosed groups and one control group, each comprising 10 rats of both sexes. The 2-yr study consisted of 3 dosed groups and one control group, each comprising 50 rats of both sexes. The rats were housed individually in stainless-steel wire hanging cages (170 mm [W] × 294 mm [D] × 176 mm [H]) under controlled environmental conditions (temperature of 23 ± 2°C and relative humidity of 55 ± 15% with 15 to 17 air changes/hr) in barrier system animal rooms. Fluorescent lighting was controlled automatically to provide a 12-h light/dark cycle. All animals had free access to filtered, UV irradiation-sterilized water supplied by an automatic watering system.

Diet preparation and feeding

For the 13-wk study, a diet containing 512, 1,280, 3,200, 8,000 or 20,000 ppm ACP (w/w) was prepared by mixing ACP with γ irradiation-sterilized CRF-1 powdered diet (Oriental Yeast Co., Tokyo, Japan) in a spiral mixer for 5 min, after which it was stored at 7°C until use. For the 2-yr study, a diet containing 1,280, 3,200 or 8,000 ppm ACP was prepared by the same method. The high dose level of 8,000 ppm was chosen, so as not to exceed the maximum tolerated dose (MTD) on the basis of the body weight gain and subchronic toxicity seen in the 13-wk study. We used the same MTD criteria as those set for 2-yr carcinogenicity studies in the guidelines of the National Cancer Institute (NCI)[17] and the IARC[18]. The powdered diet containing ACP was prepared at intervals of 2 wk and stored in a refrigerator for its use during the 13-wk and 2-yr administration periods. The feeder filled with ACP-containing or control diet in individual cages was exchanged twice a week. ACP concentrations in the powdered diet were determined by liquid chromatography, and were found to range from 93.4 to 106.0% of the target concentrations at the time of preparation. These initial concentrations decreased to 90.3 to 97.5% on the 15th day after preparation, when the concentrations at the time of preparation were taken as 100%. The animals were fed diets containing ACP at different concentrations or diet only as a control throughout the 13-wk and 2-yr administration periods, starting at the age of 6 wk.

Clinical observations and analysis, and pathological examinations

The rats were observed daily for clinical signs and mortality. Body weight and food consumption were measured once a week for the 13-wk study, and once a week for the first 14 wk of the 2-yr administration period

Table 1. Terminal Body weight, food consumption and chemical intake of male and female rats in the 13-wk feeding study of ACP

Group (ppm)	Control	512	1,280	3,200	8,000	20,000
Male						
No. of animals	10	10	10	10	10	10
Terminal body weight (g)	330 ± 16	325 ± 13	334 ± 11	332 ± 9	312 ± 17*	285 ± 10**
Food consumption (g/day)	14.2 ± 0.7	13.9 ± 0.6	14.2 ± 0.5	14.3 ± 0.4	13.6 ± 0.6	12.8 ± 0.5**
Chemical intake (g/kg bw/day)	–	0.029 ± 0.001	0.072 ± 0.001	0.184 ± 0.013	0.459 ± 0.010	1.188 ± 0.037
Female						
No. of animals	10	10 10	10	10	10	
Terminal body weight (g)	174 ± 8	178 ± 15	175 ± 12	172 ± 7	167 ± 11	161 ± 9*
Food consumption (g/day)	9.7 ± 0.4	9.7 ± 0.8	9.7 ± 0.9	9.5 ± 0.4	9.2 ± 0.6	9.0 ± 0.5
Chemical intake (g/kg bw/day)	–	0.033 ± 0.001	0.083 ± 0.004	0.205 ± 0.006	0.513 ± 0.016	1.316 ± 0.034

Mean ± SD of all animals averaged over the 13-wk administration period. * and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Dunnett's test, respectively.

and every 4 wk thereafter for the 2-yr study. All animals underwent complete necropsy. Urinary parameters were measured in the last week of the 13-wk administration period or the 2-yr administration period with Ames reagent strips (Multistix, Simens Healthcare Diagnostics, Inc., USA). For hematology and blood biochemistry, blood samples were collected under ether anesthesia at terminal necropsy after overnight fasting. Hematological parameters were measured with an Automatic Blood Cell Analyzer (ADVIA 120, Bayer Co., USA). Blood levels of methemoglobin were determined only for the 13-wk subchronic study with a CO-oximeter (CIBA-CORNING 270, Bayer Co., USA). Blood biochemical parameters were measured with an Automatic Analyzer (HITACHI 7080, Hitachi Ltd., Japan). Organs were removed, weighed and examined for macroscopic lesions. All organs and tissues indicated in the OECD test guidelines[13, 14] were examined for histopathology in all animals. The tissues for microscopic examination were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections of 5 μm in thickness were prepared, and stained with hematoxylin and eosin (H & E).

Statistical analysis

Incidences of non-neoplastic lesions and urinary data were analyzed by Chi-square test. Incidences of neoplastic lesions were statistically analyzed with Fisher's exact test. The positive trend of the dose-response relationship for the neoplastic incidences was analyzed by Peto test[19]. Body weight, food consumption, organ weights, and hematological and blood biochemical parameters were analyzed by Dunnett's test. The Kaplan-Meier method[20] and the log-rank test[21] were used to test the statistical significance of the survival rates between any ACP-fed rat of either sex and the respective control.

Results

Thirteen-week study

Survival, food consumption, chemical intake, body and organ weights and clinical signs: All ACP-fed rats of both sexes survived to the end of the 13-wk administration period. Terminal body weight was significantly decreased in the rats of both sexes fed 20,000 ppm and in the male rats fed 8,000 ppm (Table 1). Food consumption was significantly lowered only in the male rats fed 20,000 ppm (Table 1). Amounts of daily ACP intake per body weight increased proportionally at the same ratio as the stepwise increase of 2.5-fold in the ACP concentrations of the diet.

Macroscopic examination at terminal necropsy revealed that all 20,000 ppm-fed rats of both sexes had both enlarged spleen and thickened forestomach wall (Table 2). Relative weights of lungs, kidneys, spleen and liver were significantly increased in the male rats fed 3,200 ppm and above, except for the lung and spleen at 3,200 ppm. The ACP-fed female rats exhibited a statistically significant increase in relative spleen and liver weights at 8,000 and 20,000 ppm and in relative kidney weight at 20,000 ppm.

The increase in methemoglobin levels attained statistical significance in the male rats fed 8,000 and 20,000 ppm and in the 20,000 ppm-fed females (Table 2). A significant increase in reticulocyte counts was found in the male rats fed 1,280 ppm and above and in the females fed 3,200 ppm and above. Red blood cell counts (RBC) were significantly decreased in the males fed 3,200 ppm and above and in the females fed 1,280 ppm and above. Hemoglobin concentration (Hb) and hematocrit (Ht) were significantly decreased in the male and female rats fed 3,200 ppm and above, except for Ht in the 3,200 ppm-fed females. The plasma level of total bilirubin was significantly increased in the 20,000 ppm-fed rats of both

Table 2. Macroscopic findings, relative organ weights and clinical data of male and female rats in the 13-wk feeding study of ACP

Group (ppm)	Control	512	1,280	3,200	8,000	20,000
Male						
No. of animals	10	10	10	10	10	10
Macroscopic findings						
Spleen: enlarged	0	0	0	0	0	10
Forestomach: thickened	0	0	0	0	1	10
Relative organ weights						
Body weight (g)	310 ± 16	304 ± 12	313 ± 11	311 ± 9	291 ± 17**	266 ± 11**
Lungs (%)	0.311 ± 0.016	0.321 ± 0.015	0.317 ± 0.013	0.314 ± 0.009	0.333 ± 0.017**	0.343 ± 0.017**
Kidneys (%)	0.593 ± 0.014	0.599 ± 0.015	0.596 ± 0.018	0.620 ± 0.025*	0.661 ± 0.027**	0.704 ± 0.022**
Spleen (%)	0.187 ± 0.006	0.185 ± 0.006	0.191 ± 0.008	0.205 ± 0.010	0.257 ± 0.009**	0.456 ± 0.024**
Liver (%)	2.394 ± 0.093	2.418 ± 0.077	2.404 ± 0.074	2.532 ± 0.083**	2.570 ± 0.077**	2.972 ± 0.109**
Hematology						
RBC ($10^6/\mu l$)	9.34 ± 0.17	9.40 ± 0.19	9.32 ± 0.17	8.97 ± 0.13**	8.56 ± 0.17**	7.82 ± 0.15**
Hb (g/dl)	16.3 ± 0.3	16.4 ± 0.3	16.2 ± 0.2	15.6 ± 0.3**	15.3 ± 0.3**	14.6 ± 0.2**
Hematocrit (%)	45.6 ± 0.6	45.9 ± 1.0	45.5 ± 0.8	44.5 ± 0.9*	44.0 ± 0.6**	42.3 ± 0.8**
Reticulocyte counts (%)	1.8 ± 0.1	1.8 ± 0.2	2.0 ± 0.2*	2.6 ± 0.2**	3.8 ± 0.3**	5.6 ± 0.2**
Methemoglobin (%)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.8 ± 0.3**	1.0 ± 0.2**
Blood biochemistry						
Total bilirubin (mg/dl)	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.16 ± 0.02**
Female						
No. of animals	10	10	10	10	10	10
Macroscopic findings						
Spleen: enlarged	0	0	0	0	0	10
Forestomach: thickened	0	0	0	0	0	10
Relative organ weights						
Body weight (g)	160 ± 8	165 ± 15	162 ± 10	159 ± 6	154 ± 10	149 ± 9*
Lungs (%)	0.444 ± 0.023	0.450 ± 0.027	0.445 ± 0.025	0.446 ± 0.025	0.461 ± 0.024	0.468 ± 0.023
Kidneys (%)	0.664 ± 0.028	0.670 ± 0.040	0.661 ± 0.026	0.665 ± 0.027	0.689 ± 0.040	0.734 ± 0.016**
Spleen (%)	0.235 ± 0.012	0.231 ± 0.009	0.236 ± 0.011	0.257 ± 0.019	0.299 ± 0.009**	0.498 ± 0.020**
Liver (%)	2.335 ± 0.086	2.315 ± 0.080	2.390 ± 0.147	2.407 ± 0.083	2.517 ± 0.093**	2.990 ± 0.132**
Hematology						
RBC ($10^6/\mu l$)	8.76 ± 0.23	8.60 ± 0.21	8.50 ± 0.16*	8.20 ± 0.22**	7.75 ± 0.25**	7.14 ± 0.16**
Hb (g/dl)	16.3 ± 0.5	16.0 ± 0.4	15.9 ± 0.3	15.6 ± 0.4**	15.0 ± 0.4**	14.1 ± 0.3**
Hematocrit (%)	44.5 ± 1.1	43.8 ± 1.0	43.8 ± 0.6	43.6 ± 1.3	41.9 ± 1.2**	40.0 ± 0.8**
Reticulocyte counts (%)	1.7 ± 0.2	1.7 ± 0.2	2.1 ± 0.2	2.5 ± 0.4**	3.7 ± 0.7**	5.7 ± 0.7**
Methemoglobin (%)	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.7 ± 0.3	1.0 ± 0.3**
Blood biochemistry						
Total bilirubin (mg/dl)	0.14 ± 0.02	0.14 ± 0.02	0.14 ± 0.03	0.19 ± 0.14	0.15 ± 0.02	0.17 ± 0.02*

Values are expressed as mean ± SD. * and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Dunnett's test, respectively. RBC: Red blood cell counts, Hb: Hemoglobin concentration. Body weight was measured at terminal necropsy after overnight fasting.

sexes. No hematuria occurred in any group of either sex fed ACP.

Histopathology: In the forestomach, hyperplasia was observed in all rats of both sexes fed 8,000 and 20,000 ppm, while the incidence of erosion/ulcer was significantly increased in the 20,000 ppm-fed females (Table 3). The forestomach hyperplasia was characterized by diffuse proliferation of squamous cell epithelium with hyperkeratinization. Papillary projection of the hyperplastic epithelium into the stomach lumen and basal cell proliferation were observed in the severe cases of hyperplasia (Fig. 1). In the urinary bladder, the incidence of transitional cell hyperplasia was significantly increased in the 20,000 ppm-fed male rats (Table 3), and two morphologically different types of hyperplasias, i.e., simple and papillary and/or nodular types (PN), were

Table 3. Incidences of histopathological lesions of male and female rats in the 13-wk feeding study of ACP

	Male						Female					
Group (ppm)	Control	512	1,280	3,200	8,000	20,000	Control	512	1,280	3,200	8,000	20,000
No. of animals	10	10	10	10	10	10	10	10	10	10	10	10
Forestomach												
Hyperplasia	0	0	0	0	10**	10**	0	0	0	0	10**	10**
Erosion/Ulcer	0	0	0	0	0	1	0	0	0	0	0	4*
Urinary bladder												
Transitional epithelium												
Hyperplasia	0	0	0	0	0	9**	0	0	0	0	0	0
(simple)	(0)	(0)	(0)	(0)	(0)	(9)	(0)	(0)	(0)	(0)	(0)	(0)
(PN)	(0)	(0)	(0)	(0)	(0)	(6)	(0)	(0)	(0)	(0)	(0)	(0)
Swelling	0	0	0	0	0	9**	0	0	0	0	0	5*
Spleen												
Deposit of hemosiderin	0	0	0	10**	10**	10**	0	0	0	10**	10**	10**
Extramedullary hematopoiesis	0	0	0	10**	10**	10**	0	0	0	0	10**	10**
Engorgement of erythrocyte	0	0	0	10**	10**	10**	0	0	0	10**	10**	10**

* and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Chi square test, respectively. PN indicates papillary and/or nodular hyperplasia.

found (Fig. 2). An increase in the number of transitional epithelium layers to six or more layers was observed in the simple hyperplasia. The PN hyperplasia was characterized by papillary projection of the transitional cell epithelium with a fibrovascular stroma into the bladder lumen and/or by proliferation of transitional cells arranged in a solid island that appeared in the transitional epithelium or extended into the lamina propria. All the PN hyperplasias were accompanied by the simple hyperplasia. Notably, crystalline or amorphous precipitate could not be detected in the urinary bladder of male rats fed 20,000 ppm for 13 wk. Swelling of the transitional epithelium in the urinary bladder, indicating a degenerative change in the transitional cells, occurred in the 20,000 ppm-fed rats of both sexes (Table 3). Splenic lesions including hemosiderin deposition, extramedullary hematopoiesis and erythrocyte engorgement occurred with statistical significance in the rats of both sexes fed 3,200 ppm and above, except for extramedullary hematopoiesis in the 3,200 ppm-fed females (Table 3). No treatment-related lesion was observed in other organs, including the lung, kidney and liver, in the ACP-fed rat groups of either sex.

Two-year study

Survival, food consumption, body and organ weights and clinical signs: Terminal survival rates were 66%, 76%, 78% and 78% for the control, 1,280, 3,200 and 8,000 ppm-fed males and 84%, 90%, 92% and 80% for the control, 1,280, 3,200 and 8,000 ppm-fed females, respectively. These results indicate that there was no significant difference in the terminal survival rates between any ACP-fed groups of either sex and the respective control. As shown in Table 4, there was no significant difference in terminal body weight between any ACP-fed male group and the male control, but both 3,200 and 8,000 ppm-fed female rats exhibited significant decreases in terminal body weight of 7% and 13%, respectively, as compared with the female control. Food consumption was significantly lowered in the male rats fed 3,200 and 8,000 ppm and in the females fed 8,000 ppm. The 2-yr averaged amount of daily ACP intake per body weight increased proportionally at the same ratio as the stepwise increase of 2.5-fold in the ACP concentrations of the diet.

Macroscopic examination at terminal necropsy revealed a dose-dependent increase in incidences of forestomach nodules in the ACP-fed rats of both sexes (Table 4). The relative kidney weight increased dose-dependently in both sexes and attained statistical significance in the 8,000 ppm-fed males and in the females fed 3,200 and 8,000 ppm. No dose-related increase in the relative weight of lungs, spleen or liver was found in either ACP-fed males or females.

Except for a clear decrease in RBC in the 8,000 ppm-fed females (Table 4), no dose-related changes in the hematological or blood biochemical parameters were found in any group of either sex fed 1,280, 3,200 or 8,000 ppm, as compared with the respective control (data not shown). Indeed, the female RBC value was greater at 3,200 ppm than at 1,280 ppm, suggesting no dose-response relationship at low dose levels. No hematuria

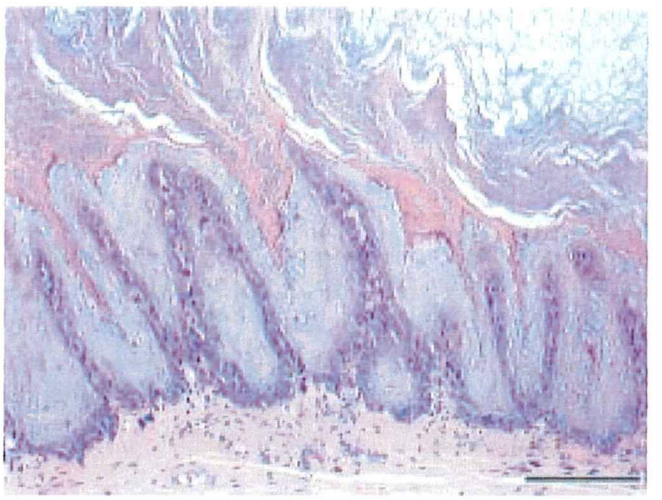

Fig. 1. Squamous cell hyperplasia in the forestomach of a male rat fed 20,000 ppm ACP for 13 wk. Bar indicates 200 μm. H & E stain.

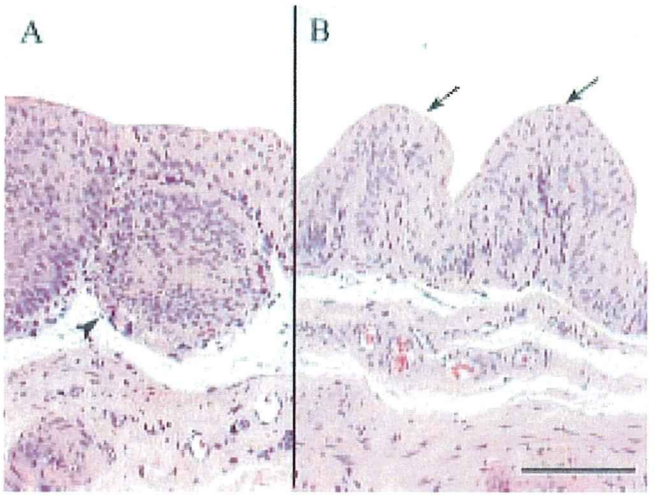

Fig. 2. Papillary and/or nodular hyperplasia of transitional epithelium in the urinary bladder of a male rat fed 20,000 ppm ACP for 13 wk. An arrowhead indicates the nodular nest of the transitional cell epithelium in the lamina propria (A). Arrows indicate the papillary projection of the transitional cell epithelium with a fibrovascular stroma (B). Bar indicates 200 μm. H & E stain.

occurred in any ACP-fed group of either sex.

Histopathology: As indicated by Fisher's exact test, statistically significant increases in incidences of forestomach squamous cell papillomas were noted in the male rats fed 3,200 and 8,000 ppm and in the 8,000 ppm-fed female rats, while the incidence of squamous cell carcinomas was significantly increased in the 8,000 ppm-dosed male rats (Table 5). The incidences of forestomach tumors increased dose-dependently as indicated by a significant positive trend by Peto test. The incidence of transitional cell carcinomas in the urinary bladder increased dose-dependently only in male rats and attained statistical significance at 8,000 ppm. These two malignant tumors did not metastasize to any other organ. The

Table 4. Terminal body weight, food consumption, chemical intake, macroscopic finding, relative organ weight and hematological data of male and female rats in the 2-yr feeding study of ACP

Group (ppm)	Control	1,280	3,200	8,000
Male				
No. of animals used	50	50	50	50
No. of surviving animals[a)]	33	38	39	39
Terminal body weight (g)[b)]	410 ± 43	414 ± 36	423 ± 40	390 ± 38
Food consumption (g/day)[c)]	15.7 ± 1.2	15.3 ± 1.0	15.2 ± 0.9*	15.1 ± 1.0*
Chemical intake (g/kg bw/day)[c)]	–	0.056 ± 0.004	0.144 ± 0.006	0.373 ± 0.020
Macroscopic finding				
Forestomach: nodule	0	3	9	41
Relative organ weight				
Body weight(g)[d)]	390 ± 43	394 ± 35	403 ± 38	371 ± 38
Kidneys (%)	0.773 ± 0.140	0.759 ± 0.119	0.783 ± 0.093	0.927 ± 0.282**
Hematology				
RBC ($10^6/\mu l$)	7.56 ± 1.25	8.14 ± 1.14*	8.43 ± 0.74**	7.88 ± 0.89
Female				
No. of animals used	50	50	50	50
No. of surviving animals[a)]	42	45	46	40
Terminal body weight (g)[b)]	293 ± 32	294 ± 34	273 ± 31*	254 ± 32**
Food consumption (g/day)[c)]	11.3 ± 1.1	11.6 ± 1.1	11.0 ± 1.1	10.6 ± 0.9**
Chemical intake (g/kg bw/day)[c)]	–	0.075 ± 0.005	0.184 ± 0.013	0.469 ± 0.027
Macroscopic finding				
Forestomach: nodule	1	1	5	32
Relative organ weight				
Body weight (g)[d)]	277 ± 33	280 ± 32	259 ± 31*	240 ± 31**
Kidneys (%)	0.674 ± 0.097	0.668 ± 0.076	0.708 ± 0.090*	0.778 ± 0.153**
Hematology				
RBC ($10^6/\mu l$)	8.03 ± 0.78	7.81 ± 0.92	7.91 ± 0.66*	7.62 ± 0.31**

* and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Dunnett's test, respectively.

a: No. of surviving animals at the end of the 2-yr administration period. b: Mean ± SD of surviving animals averaged at the end of the 2-yr administration period. c: Mean ± SD of the values measured at intervals of 1 wk for the first 14 wk and 4 wk thereafter and averaged over the 2-yr administration period. d: Mean ± SD of body weights which were mesured at terminal necropsy after overnight fasting.

diagnostic criteria of squamous cell tumors in the forestomach and transitional cell tumor in the urinary bladder were based on the International Classification of Rodent Tumours[22)] and Monographs on Pathology of Laboratory Animals[23, 24)] and on Guides for Toxicologic Pathology[25)], respectively. The forestomach squamous cell papilloma was characterized by exophytic growth of squamous epithelium with fibrovascular stroma arranged in branched finger-like processes. The squamous cell carcinomas were morphologically well differentiated and of keratinized type (Fig. 3). The malignant tumor was defined as invasive growth into the lamina propria or submucosal tissue. The malignant forestomach tumor was often accompanied by ulcers. The transitional cell carcinoma in the urinary bladder projected into the lumen as an exophytic growth, which was characterized by multilayer formation with irregular growth (Fig. 4). Notably, crystalline or amorphous precipitate such as calculus could not be detected in the urinary bladder of the 8,000 ppm-fed male rats bearing transitional cell carcinoma. Significantly increased incidences of hyperplasia were noted in the forestomach of male rats given 3,200 and 8,000 ppm and in the female rats given 8,000 ppm, whereas only two cases of PN hyperplasia were observed in the urinary bladder of 8,000 ppm-fed male rats (Table 5). Neither statistically significant nor dose-dependent increase in the incidence of erosion/ulcer was observed in any ACP-fed rat of either sex. Neither statistically significant nor dose-dependent increase in the incidences of hemosiderin deposition, extramedullary hematopoiesis or erythrocyte engorgement in the spleen was noted in any ACP-fed rat, except for the 8,000 ppm-

Table 5. Incidences of non-, pre- and neoplastic lesions of male and female rats in the 2-yr feeding study of ACP

	Male					Female				
Group (ppm)	Control	1,280	3,200	8,000	Peto	Control	1,280	3,200	8,000	Peto
No. of animals	50	50	50	50	test	50	50	50	50	test
Forestomach										
Squamous cell papilloma	0	2	11**	39**	↑↑	1	1	1	25**	↑↑
Squamous cell carcinoma	0	0	0	12**	↑↑	0	0	0	2	
Combined incidence[a)]	0	2	11**	43**	↑↑	1	1	1	25**	↑↑
Hyperplasia	4	3	14#	45##		2	2	9	40##	
Erosion/Ulcer	5	1#	4	3		1	1	1	3	
Urinary bladder										
Transitional cell papilloma	0	0	1	0		0	0	0	0	
Transitional cell carcinoma	0	0	0	7**	↑↑	0	0	0	0	
Combined incidence[b)]	0	0	1	7**	↑↑	0	0	0	0	
Transitional epithelium										
Hyperplasia	0	1	0	2		0	0	0	0	
(PN)	(0)	(0)	(0)	(2)		(0)	(0)	(0)	(0)	

* and **: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Fisher's exact test, respectively. ↑ and ↑↑: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Peto test, respectively. # and ##: Significantly different at $p \leq 0.05$ and $p \leq 0.01$ by Chi-square test, respectively. a: Combined incidence of squamous cell papilloma and carcinoma. b: Combined incidence of transitional cell papilloma and carcinoma. PN indicates papillary and/or nodular hyperplasia.

fed females which exhibited slightly increased severity of hemosiderin deposition (data not shown). No significant increase in the incidence of neoplastic or non-neoplastic lesions was found in the other organs, including the kidney, of the ACP-fed rat groups of either sex.

Discussion

Subchronic toxicity and preneoplastic lesions

It was found in the present 13-wk study that the subchronic toxicity of ACP was characterized by proliferative lesions of hyperplasia in both the forestomach and urinary bladder and by erythrocyte toxicity as evidenced by decreases in the erythrocyte parameters and concurrent increases in reticulocyte counts and methemoglobin level.

A principal characteristic of the subchronic toxicity was induction of the proliferative lesion leading to development of neoplasm, which occurred in the forestomach of male and female rats fed 8,000 and 20,000 ppm and in the urinary bladder of 20,000 ppm-fed male rats. Histopathological examination revealed that there were two different types (simple and PN) of hyperplasia in the transitional epithelium of the urinary bladder. Consistently, Fukushima *et al.*[26] reported that 4-wk treatment of rats with an urinary bladder carcinogen, N-butyl-N-(4-hydroxybutyl)nitrosamine, induced an irreversible type of PN hyperplasia in the urinary bladder, which led to development of an urinary bladder tumor. Therefore, the PN hyperplasia found in the urinary bladder of male rats fed ACP for 13 wk is considered to develop to the urinary bladder tumor after prolonged oral administration of ACP in feed up to 2 yr. It was noteworthy, however, that crystalline or amorphous precipitate, as a causative factor that would mechanically damage the transitional epithelium, could not be found in the urinary bladder following either 13-wk or 2-yr administration of ACP. Non-neoplastic lesions were observed only at 20,000 ppm as erosion/ulcer in the forestomach and as swelling of transitional epithelium in the urinary bladder.

Another characteristic of the ACP-induced subchronic toxicity was induction of the erythrocyte toxicity which was accompanied by hemosiderin deposition, extramedullary hematopoiesis and erythrocyte engorgement in the spleen of ACP-fed rats. These splenic lesions are considered to reflect the morphological correlates of enhanced splenic function for scavenging the damaged erythrocytes. The most sensitive sign of erythrocyte toxicity was manifested as the compensatorily increased reticulocyte counts in the 1,280 ppm-fed males, although the methemoglobin level was significantly increased only in the males fed 8,000 and 20,000 ppm and in the females fed 20,000 ppm. Tomoda *et al.*[8] reported that two anodic components of hemoglobins were markedly enhanced in comparison with the increased level of methemoglobin in the erythrocytes of workers handling

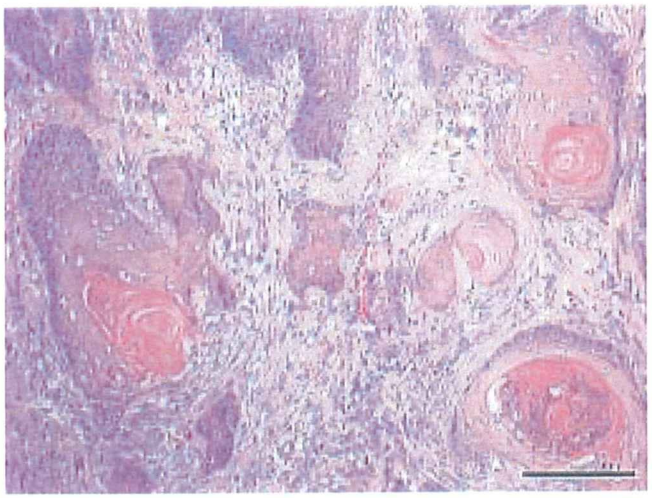

Fig. 3. Squamous cell carcinoma in the forestomach of a male rat fed 8,000 ppm ACP for 2 yr. Bar indicates 200 μm. H & E stain.

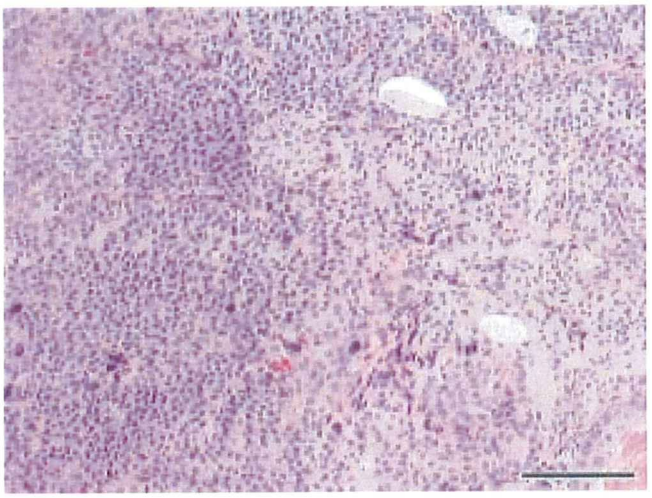

Fig. 4. Transitional cell carcinoma in the urinary bladder of a male rat fed 8,000 ppm ACP for 2 yr. Bar indicates 200 μm. H & E stain.

aromatic compounds including ACP, suggesting possible oxidation and modification of intracellular hemoglobins other than the methemoglobin formation. Therefore, taken together with the large difference in the statistically significant dose level between the reticulocyte counts and the methemoglobin, it remains to be solved whether or not the formation of methemoglobin is a primary cause of the ACP-induced erythrocyte toxicity. Notably, the erythrocyte toxicity appeared at lower dose levels in the present 13-wk study than the two preneoplastic and two non-neoplastic lesions did.

Carcinogenicity and chronic toxicity

Two-yr oral administration of ACP in feed was found to significantly increase the incidences of forestomach squamous cell papillomas in the male rats fed 3,200 and 8,000 ppm and in the 8,000 ppm-fed female rats, and forestomach squamous cell carcinomas in the 8,000 ppm-fed males, as well as the incidences of urinary bladder transitional cell carcinomas in the 8,000 ppm-fed males. The increases in the incidences of the two types of tumor and their combined incidences were dose-dependent as judged by a significantly positive trend according to Peto

test. It was noteworthy that the organs in which the two tumors were induced were exactly the same as those in which hyperplasias were induced following 13-wk administration of ACP in feed. These results suggest that the proliferative lesions of hyperplasias that occurred in the forestomach and urinary bladder after the 13-wk administration of ACP progress from benign tumors to malignant ones in the course of prolonged administration of ACP in feed extending up to 2 yr. The morphological examination revealed the invasive growth in the forestomach squamous cell carcinomas and the multilayer formation with irregular growth in the urinary bladder transitional cell carcinomas, both of which were diagnosed as being malignant[22, 23]. However, the malignant tumors found in these two organs did not metastasize to any other organ. The significantly increased incidences of forestomach hyperplasias were noted at the same dose levels as those of forestomach tumors. On the other hand, only two cases of PN hyperplasia in the transitional epithelium were noted in the males fed ACP at 8,000 ppm for 2 yr.

The high dose level of 8,000 ppm used in the present 2-yr study was selected, predicting the MTD on the basis of both the body weight change and the subchronic toxicity in the 13-wk study. The high dose level was found to fulfill the MTD criteria set by NCI[17] and IARC[18] that the highest dose of the test agent given during the carcinogenicity study should not exceed the MTD that can be predicted to elicit signs of minimal toxicity without reducing the animals' normal longevity from toxic effects other than carcinogenicity, or no more than a 10% weight decrement, as compared to the concurrent controls. The present 2-yr study, terminal body weights of 8,000 ppm-fed male and female rats were decreased by 5 and 13%, respectively, compared with the respective controls, and no significant difference in the terminal survival rate was found between any ACP-fed group of either sex and the respective control. It is considered that the recommendation regarding the 10% decrease in the body weight gain is just empirical, and that even if the decrease in body weight gain exceeded 10% in practice, the 2-yr carcinogenicity bioassay results would not be invalidated[27]. Therefore, fulfillment of the MTD criteria for the present dose levels would allow extrapolation of the present dose-carcinogenic response relationships to responses that are expected to occur at lower dose levels of environmental relevance.

It was found in the present 2-yr and 13-wk studies that clear signs of chronic erythrocyte toxicity were not evident after the 2-yr administration of ACP at 8,000 ppm and below except for the decreased RBC in the 8,000 ppm-fed female rats, whereas significant changes in the erythrocyte parameters were observed following the 13-wk administration of ACP in the males fed 3,200 ppm and above and in the females fed 1,280 ppm and above. Consistently, our previous studies[28, 29] showed the same attenuated response of erythrocytes to a potent hematotoxicant, para-chloronitrobenzene (*p*-CNB) by prolonged administration of *p*-CNB to rats from 13 wk to 2 yr. Therefore, it might be rational to infer from these results that prolonged administration of ACP in rats induced some adaptive change in erythrocytes such as formation of resistance to erythrocyte injury by hematotoxic substances, regardless of the mechanisms involved.

Genotoxicity

Our JBRC laboratory reported the data of bacterial mutagenicity and mammalian chromosome aberration for ACP[10, 11]. The bacterial mutagenicity of ACP was positive with TA100 and TA1537, only when activated with S9[10]. The chromosomal aberration assay with Chinese hamster lung cells (CHL/IU) was positive only for structural aberration without S9 activation and markedly positive for both structural and numerical aberrations including increased number of polyploid cells, when activated with S9[10]. These results can be taken to indicate that metabolites of ACP formed by the liver xenobiotic enzymes are more potently mutagenic than ACP per se. Therefore, it can be inferred that the ACP-induced carcinogenicity found in the present 2-yr study is causally related to fixing the tumor-initiating DNA damage by ACP metabolites into mutations. Furthermore, the increased number of polyploid cells in the CHL/IU chromosomal aberration assay with S9 activation[10] suggests a possibility of aneuploid cells leading to genomic instability as a causative factor for ACP-induced carcinogenesis. Therefore, a genotoxic mode of action is thought to operate in ACP-induced carcinogenesis rather than a non-genotoxic mechanism such as a cytotoxic-proliferative mode of action[30].

Extrapolation to human cancer risk

Humans do not have the forestomach, and consideration should be given to the use of rodent forestomach tumor data for predicting the cancer risk in humans. Procter *et al.*[31] proposed that a weight of evidence decision criteria for use of rodent forestomach tumor data for classification of potential human carcinogenicity and cancer risk assessment included genotoxicity induction of tumors not only in the forestomach but also in the other organs, oral administration not by gavage but by drinking or feeding, and fulfillment of MTD criteria for the high dose level. Since ACP and its present carcinogenicity study fulfilled these four criteria, the ACP-induced forestomach tumors of rats were judged to be applicable to humans.

Relevance for extrapolation of the rat urinary bladder tumors to humans has also been argued[32], since close association between chemically induced rodent urinary

bladder carcinogenicity and calculus formation has been established[33–36], and since the formation of calculus by biphenyl is considered to be species- and sex-specific[37,38]. In addition to the reported findings of positive mutagenicity of ACP[9–11], the present 13-wk and 2-yr studies confirmed that neither the crystalline or amorphous precipitate in the urinary bladder nor the hematuria, possibly resulting from the mechanical damage of transitional epithelium by the precipitate, occurred in any ACP-fed rat of either sex. It can be inferred, therefore, that a genotoxic mode of action without formation of crystalline or amorphous precipitate in the urinary bladder operates in ACP-induced urinary bladder carcinogenesis. It is interesting to note that in the present study, the occurrence of transitional cell tumors in the urinary bladder in the 2-yr study as well as hyperplasias in the same tissue in the 13-wk study was much more pronounced in male than in female rats, although the amount of daily intake of ACP per body weight was greater in females than in males. These present findings are essentially consistent with the report that the male:female ratio for the incidence of transitional cell tumors in the urinary bladder is approximately 3:1 in humans[39]. ACP-induced carcinogenicity including male predominance of bladder tumors in rats will warrant further mechanistic investigation with the ACP-fed rat, in addition to urgent need for epidemiological studies on exposure of humans to ACP and its related compounds in workplaces.

Conclusion

The 13-wk study showed the occurrence of preneoplastic lesions of hyperplasias in both the forestomach and urinary bladder and clear manifestation of erythrocyte toxicity in the ACP-fed rats. The present 2-yr study provided novel information about the carcinogenicity of ACP as clearly evidenced by the dose-dependent and significant increases in the incidences of forestomach squamous cell papillomas and carcinomas in male and female rats, and urinary bladder transitional cell carcinomas in male rats following 2-yr administration of ACP in feed. Clear signs of chronic erythrocyte toxicity disappeared after prolonged administration of ACP-containing diet for 2 yr.

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Original Article

Enhanced hepatocarcinogenicity by combined inhalation and oral exposures to *N,N*-dimethylformamide in male rats

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ABSTRACT — *N,N*-Dimethylformamide (DMF), a ubiquitous contaminant in living and working environments, enters the human body by inhalation, as well as by oral and dermal routes of exposure. In order to provide bioassay data for carcinogenic risk assessment of humans exposed to DMF by multiple routes of exposure, hepatocarcinogenic effect of combined inhalation and oral exposures of rats to DMF was examined. A group of 50 male F344 rats, 6-week-old, was exposed by inhalation to 0 (clean air), 200, or 400 ppm (v/v) of DMF vapor-containing air for 6 hr/day and 5 days/week during a 104-week period, and each inhalation group was given *ad libitum* DMF-formulated drinking water at 0, 800 or 1,600 ppm (w/w) for 104 weeks. Incidences of hepatocellular adenomas and carcinomas and their combined incidences were significantly increased in the combined-exposure groups compared with the untreated control group or each of the inhalation-alone and oral-alone groups with matching concentrations. Incidences of hepatocellular adenomas and carcinomas induced by the combined exposures were greater than the sum of the two incidences of the hepatocellular adenomas and carcinomas induced by the single-route exposures through inhalation and ingestion. The combined exposures enhanced tumor malignancy. It was concluded that the combined inhalation and oral exposures markedly enhance the incidences and malignancy of hepatocellular tumors, suggesting that the hepatocarcinogenic effect of the combined exposures is greater than the effect that would be expected under the assumption that the two effects of single-route exposures through inhalation and drinking are additive.

Key words: *N,N*-Dimethylformamide, Combined exposure, Inhalation exposure, Rat, Hepatocarcinogenicity

INTRODUCTION

N,N-Dimethylformamide (DMF) has been widely used as a solvent for acrylic fibers and polyurethanes and as a chemical intermediate in chemical manufacturing and pharmaceutical industries (IPCS, 1991). The worldwide production capacity of DMF was estimated to be 125,000 tons /year (IARC, 1999). The annual production of DMF in Japan was reported to amount to 50,000 tons in 2006 (Chemical Daily, 2008). According to U.S. EPA's Toxics Release Inventory Report (2006), total On- and Off-site Disposal or Other Release for DMF amounted to 2,300,000 lbs (1,043 tons). DMF has been observed in ambient air near a fiber plant and in water near waste facilities. Air levels of 0.024 - 0.15 mg DMF/m^3 (0.008 - 0.05 ppm (v/v)) collected in a residential area were reported to originate from neighboring industry (Amster *et al.*, 1983). Concentrations of DMF in public waters were reported to range from 0.037 to 1.5 ppb (w/w), according to a report by the Japan Ministry of the Environment (2006). Ubiquitous presence of DMF in urban air and public waters suggests that the general population may be exposed to DMF at low levels through inhalation and ingestion. On the other hand, workers using DMF in workplaces are at high health risk of exposure to high levels of DMF primarily through inhalation and dermal contact. According to the National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health (NIOSH, 1990), a total of 124,683 workers including 16,011 females were potentially exposed to DMF in workplaces in the USA between 1981 and 1983. Workplace air concentrations of DMF in a textile factory in the

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UK were reported to range from 0.36 to 7.00 ppm (v/v) (1.1 to 21.0 mg/m^3) (Osunsanya *et al.*, 2001).

The International Agency for Research on Cancer (IARC, 1999) made an overall evaluation that DMF is not classifiable as to its carcinogenicity to humans (Group 3), based on both inadequate evidence in humans for carcinogenicity of DMF and evidence suggesting a lack of the carcinogenicity in experimental animals. Malley *et al.* (1994) reported no evidence of carcinogenicity after 2-year inhalation exposure of rats or mice to DMF, and their finding was adopted for the IARC's evaluation. More recently, however, Senoh *et al.* (2004) have demonstrated that 2-year inhalation exposure to DMF produces hepatocellular adenomas and carcinomas in rats and mice and hepatoblastomas in mice. A broad range of *in vitro* and *in vivo* genotoxicity assays showed that genotoxicity of DMF is negative (IARC, 1999). Thus, a non-genotoxic-cytotoxic-proliferative mode of action may be hypothesized for the DMF-induced hepatocarcinogenesis, suggesting that hepatocellular death by toxic insult and subsequent regenerative proliferation of hepatocytes play a crucial role in DMF-induced hepatocarcinogenesis (Butterworth *et al.*, 1992).

It is important to understand to what extent combined exposures of a general population to low levels of environmentally ubiquitous DMF through both inhalation and ingestion enhance hepatocarcinogenic risks. However, it is not yet known whether combined inhalation and oral exposures to DMF in multiple media affect carcinogenicity additively or in a greater than additive manner. Recently, we found that combined inhalation and oral exposures of male rats to DMF for 4 weeks enhanced the proliferative response of hepatocytes in a greater than additive manner (Ohbayashi *et al.*, 2008). The aim of this bioassay study was to provide dose-carcinogenic response data for assessing carcinogenic risks in humans exposed to the environmentally ubiquitous DMF present in multiple media through multiple exposure routes. For this purpose, we examined carcinogenicity by exposing male F344 rats to DMF for 104 weeks by a single route of inhalation in the vapor phase, by oral administration in drinking water or by combined inhalation and oral exposures.

MATERIALS AND METHODS

Chemicals

Reagent-grade DMF (> 99.5%) obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) was used in this study. Gas chromatographic analysis showed that no peaks corresponding to impurities were detected in air containing 200 or 400 ppm (v/v) DMF vapor or in DMF-formulated drinking water containing 800 or 1,600 ppm (w/w).

Animals

Four-week-old, male F344/DuCrlCrj rats (SPF) were purchased from Charles River Japan, Inc. (Atsugi, Japan). Male rats were chosen in this study, since it was found in our 2-year inhalation study (Senoh *et al.*, 2004) that male rats were more sensitive to the DMF-induced hepatocarcinogenicity than female rats. The experiment was started when the rats were 6 weeks old, after 2-week quarantine and acclimation. The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (NRC, 1996), and this study was approved by the ethics committee of Japan Bioassay Research Center.

Exposure to DMF

The rats were housed individually in stainless steel wire hanging cages in an inhalation exposure chamber maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 15%, with 12 air changes/hr. The inner volume of the exposure chamber was 4.3 m^3. Nine inhalation exposure chambers were installed in two barrier system animal rooms. Fluorescent lighting was controlled automatically to give a 12-hr light/dark cycle. The rats had free access to a γ-irradiation-sterilized commercial pellet diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan).

The techniques for generating the DMF vapor-air mixture and the system for the combined inhalation and oral exposures to DMF are described in detail in a previous paper (Kano *et al.*, 2002). The study design for the combined exposures and the single-route exposure to DMF through either inhalation or ingestion is presented in Table 1. A group of 50 rats was exposed by inhalation to 200 or 400 ppm (v/v) of DMF vapor-containing air for 6 hr/day and 5 days/week for 104 weeks, and each inhalation group was given DMF-formulated drinking water at 800 or 1,600 ppm (w/w) or vehicle drinking water *ad libitum* for 24 hr/day and 7 days/week, during a 104-week period. A group of 50 rats exposed to clean air together with oral administration of the vehicle drinking water served as an untreated control (Inh-0+Orl-0 ppm group). In addition to the untreated control group, therefore, the following eight different combinations of exposures to DMF were employed in this study: Four combined-exposure groups consisted of inhalation exposure to 200 ppm with oral administration of 800 ppm drinking water (Inh-200+Orl-800 ppm), Inh-200+Orl-1,600 ppm, Inh-400+Orl-800 ppm and Inh-400+Orl-1,600 ppm. Two oral-alone groups consisting of Inh-0+Orl-800 ppm and Inh-0+Orl-1,600 ppm received oral administration of 800

Table 1. An experimental design and time-averaged concentrations of DMF in the inhalation chamber and in the DMF-formulated drinking water

Group Name	Target concentration		Observed concentration (mean ± S.D.)	
	Inhalation (ppm(v/v))	Drinking water (ppm(w/w))	Inhalation (ppm(v/v))	Drinking water (ppm(w/w))
Inh-0+Orl-0 ppm	0	0	0	-
Inh-0+Orl-800 ppm	0	800	0	796 ± 22
Inh-0+Orl-1,600 ppm	0	1,600	0	1,604 ± 46
Inh-200+Orl-0 ppm	200	0	201.3 ± 2.0	-
Inh-200+Orl-800 ppm	200	800	201.8 ± 2.9	800 ± 22
Inh-200+Orl-1,600 ppm	200	1,600	199.7 ± 1.7	1,620 ± 35
Inh-400+Orl-0 ppm	400	0	400.0 ± 3.3	-
Inh-400+Orl-800 ppm	400	800	401.3 ± 2.8	805 ± 24
Inh-400+Orl-1,600 ppm	400	1,600	400.7 ± 2.9	1,616 ± 36

and 1,600 ppm DMF-formulated water without inhalation exposure, respectively. Two inhalation-alone groups consisting of Inh-200+Orl-0 ppm and Inh-400+Orl-0 ppm were exposed by inhalation to 200 and 400 ppm DMF vapor, respectively. Means ± SDs of the observed concentrations of DMF in the inhalation exposure chamber and in the drinking water are also given in Table 1. The selection of dose levels of DMF in inhalation exposure and drinking water was based on our previous findings. First, 2-year inhalation exposure to 400 ppm induced hepatocellular adenomas without hepatocellular carcinomas, while the low concentration of 200 ppm did not increase the incidence of hepatocellular tumors (Senoh *et al.*, 2004). Second, oral administration of 1,600 ppm-formulated drinking water for 4 weeks induced a significant increase in proliferation index of proliferating cell nuclear antigen (PCNA)-positive hepatocytes, while the water concentration of 800 ppm did not increase the number of PCNA-positive hepatocytes. In addition, 4-week combined exposures to 400 ppm in inhalation and 1,600 ppm in water significantly increased the proliferation index of PCNA-positive hepatocytes (Ohbayashi *et al.*, 2008).

Clinical observations, analysis and pathologic examinations

The rats were observed daily for clinical signs and mortality. Body weights and consumption of food and water were measured weekly for the first 14 weeks, every 4 weeks thereafter and at the final 104th week. All rats that died or were killed in a moribund state during the 2-year period, and survived to the end of the 2-year period received complete necropsy. All organs and tissues indicated in the OECD test guideline (OECD, 1981) and the entire respiratory tract including nasal cavity, pharynx and larynx were examined for histopathology in all rats. The organs and tissues for histopathological examination were fixed in 10% neutral buffered formalin and embedded in paraffin. All organs and tissues were cut into 5 μm-thick sections and stained with hematoxylin and eosin (H & E).

Statistical analysis

Statistical comparison was performed between the following groups; a) each DMF-treated group vs. untreated control group; b) each of the two combined-exposure groups vs. each of the oral-alone group with matching concentration; and c) each of the two combined-exposure groups vs. each of the inhalation-alone groups with matching concentrations. We used Dunnett's test for body weights and chi-square test for incidences of neoplastic lesions and survival rates. Two-tailed test was used for all statistics, and in all cases, a *P*-value of 0.05 was used as the level of significance.

RESULTS

Survival, body weight, clinical and macroscopic observations

There was no statistically significant difference in the survival rate at any time point between the untreated control group and the two inhalation-alone groups, the two oral-alone groups or the four combined-exposure groups (Table 2). The primary cause of deaths was diagnosed on

Table 2. Survival rate, body weight, daily water consumption and estimated amounts of DMF Uptake

Group Name	Initial animal No.	No. of surviving animals (week after administration)						Terminal body weight (g) (mean ± S.D.)	Time-averaged water consumption (g/day/rat) (mean ± S.D.)	Estimated amount of DMF uptake (mg/kg/day)		
		54	74	84	94	99	104			Inhalation	Drinking water	Total
Inh-0+Orl-0 ppm	50	50	49	47	45	43	41	410 ± 67	19.4 ± 3.1	0	0	0
Inh-0+Orl-800 ppm	50	50	46	43	40	40	34	369 ± 41 *a*	19.0 ± 2.5	0	44	44
Inh-0+Orl-1,600 ppm	50	50	48	47	43	43	40	346 ± 44 *a*	17.1 ± 1.8	0	82	82
Inh-200+Orl-0 ppm	50	50	47	44	40	39	36	378 ± 26	19.3 ± 1.8	121	0	121
Inh-200+Orl-800 ppm	50	49	45	43	36	36	36	359 ± 18 *a,c*	17.4 ± 1.1	121	44	165
Inh-200+Orl-1,600 ppm	50	49	49	46	42	42	41	333 ± 29 *a,c*	15.9 ± 0.6	121	84	205
Inh-400+Orl-0 ppm	50	49	48	46	38	38	37	324 ± 41 *a*	17.1 ± 1.4	242	0	242
Inh-400+Orl-800 ppm	50	50	50	49	46	45	43	313 ± 20 *a,b,c*	16.1 ± 1.5	242	47	289
Inh-400+Orl-1,600 ppm	50	50	47	47	43	41	38	290 ± 29 *a,b,c*	15.8 ± 1.4	242	96	338

Symbols *a*, *b* and *c*: significantly different from the untreated control group, and each of the oral-alone and inhalation-alone groups with matching concentrations, respectively, at $p < 0.05$ by Dunnett's test.

the basis of the macroscopic and microscopic findings, and the number of animals that died of liver tumors was 2/50 cases for the Inh-200+Orl-1,600 ppm, 1/50 for the Inh-400+Orl-0 ppm, 4/50 for the Inh-400+Orl-800 ppm and 4/50 for the Inh-400+Orl-1,600 ppm (Table 3). This result revealed that the incidence of liver tumor deaths tended to increase in a dose-related manner, although those increased incidences were not statistically significant. The terminal body weight was significantly decreased in the inhalation-alone group (Inh-400+Orl-0 ppm), both oral-alone groups and all four combined-exposure groups, compared with the untreated control group (Table 2). Water consumption was clearly decreased in the Inh-0+Orl-1,600 ppm group, in the Inh-400+Orl-0 ppm group and in all four combined-exposure groups, compared with the untreated control group or the inhalation-alone groups with matching concentrations (Table 2). No overt clinical sign was observed in any group of DMF-treated rats throughout the 104-week period.

Estimated amounts of DMF uptake into the body are given in Table 2. The uptake of DMF through inhalation was calculated on the basis of the respiratory volume per body weight and the chamber concentration of DMF, assuming 561 ml/min for minute volume per kg body weight (Mauderly *et al.*, 1979) and the lung absorption ratio of DMF as 100% (adopted because of no data available from the literature). For instance, the estimated amount of DMF uptake for 6-hr inhalation exposure to 200 ppm resulted in 121 mg/kg/day by the following calculation (561 ml/min/kg body weight x 60 min x 6 hr/day x 600 mg/m^3 (200 ppm)). Amount of DMF uptake by the oral route was estimated by the following calculation (the daily amount of drinking water consumption x the observed water concentration of DMF/body weight x the gastrointestinal absorption ratio as 100% (adopted because of no data available from the literature)). These three parameters were obtained on a weekly basis, and the weekly multiplied values were averaged over 104 weeks, e.g., the estimated amount of DMF uptake by oral administration of 800 ppm-formulated drinking water resulted in 44 mg/kg/day. Thus, the total amount of DMF uptake in the Inh-200+Orl-800 ppm group was estimated to be 165 mg/kg/day.

Hepatocellular tumors

Table 3 shows that incidences of hepatocellular adenomas and carcinomas and their combined incidences were significantly increased in the four combined-exposure groups, the two inhalation-alone groups and the two oral-alone groups, as compared with the untreated control group and each of the oral-alone and inhalation-alone groups with matching concentrations. As a whole, those incidences of DMF-induced hepatocellular tumors were increased with an increase in total estimated amounts of DMF uptake. It should be noted, however, that the incidences of hepatocellular tumors induced by the combined-exposures tended to exceed the dose-response relation that would be expected under the assumption that the two incidences of hepatocellular tumors induced by the single-route exposures through inhalation and ingestion are additive. As an example, the incidences of hepatocellular adenomas and carcinomas in the two combined-exposure groups of Inh-200+Orl-800 ppm (total estimated uptake of 165 mg/kg/day) and Inh-200+Orl-1,600 ppm (205 mg/kg/day) were greater than those in the single-route exposure group of Inh-400+Orl-0 ppm (242 mg/kg/day), although the total estimated uptakes in the former two groups were less than that in the latter group. Furthermore, the combined exposures were found to produce multiple occurrences of hepatocellular adenomas as compared with the single-route exposures (data not shown).

The incidences of hepatocellular tumors in the combined-exposure groups were compared with the sum of the incidences of hepatocellular tumors in the single-route exposure groups through inhalation and ingestion (Fig. 1) under the same concept as "response additivity" applied to the effects of chemical mixture by the U.S. EPA (2000). The incidences of hepatocellular adenomas in the four combined-exposure groups were greater than the sum of the incidences of hepatocellular adenomas in the single-route exposure groups through inhalation and ingestion (Fig. 1A). Hepatocellular carcinomas induced by single-route exposure through either inhalation or ingestion occurred at low incidences: 1/50 case (2%) for the Inh-200+Orl-0 ppm group, 2/50 cases (4%) for the Inh-400+Orl-0 ppm group and 4/50 cases (8%) for the Inh-0+Orl-1,600 ppm group. As compared with the low incidences of hepatocellular carcinomas induced by the single-route exposures, however, the combined exposures were found to produce significantly increased incidences of hepatocellular carcinomas: 6/50 cases (12%) for the Inh-200+Orl-800 ppm, 14/50 cases (28%) for the Inh-200+Orl-1,600 ppm, 12/50 cases (24%) for the Inh-400+Orl-800 ppm, and 14/50 cases (28%) for the Inh-400+Orl-1,600 ppm. Notably, the incidences of hepatocellular carcinomas in the four combined-exposure groups were greater than the sum of the two incidences of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1B). Comparison of Fig. 1B with Fig. 1A reveals that the increased incidences of hepatocellular carcinomas that are more malignant than adenomas in the combined-exposure groups

Table 3. Number of male rats bearing hepatocellular tumors following combined inhalation and oral exposures or single-route exposures to DMF

Inhalation (ppm)	0			200			400		
Drinking water (ppm)	0	800	1,600	0	800	1,600	0	800	1,600
Total estimated amount of DMF uptake (mg/kg/day)	(0)	(44)	(82)	(121)	(165)	(205)	(242)	(289)	(338)
Number of animals examined	50	50	50	50	50	50	50	50	50
Number of animals dead or found in a moribund state	9	16	10	14	14	9	13	7	12
Hepatocellular adenoma	1	6 [a]	8 [a]	15 [a]	28 [a,b,c]	45 [a,b,c]	26 [a]	43 [a,b,c]	46 [a,b,c]
	(0)	(2)	(2)	(2)	(1)	(4)	(3)	(3)	(9)
Hepatocellular carcinoma	0	0	4 [a]	1	6 [a,b,c]	14 [a,b,c]	2	12 [a,b,c]	14 [a,b,c]
	(0)	(0)	(0)	(0)	(0)	(1)	(0)	(1)	(2)
Hepatocellular adenoma + carcinoma	1	6 [a]	12 [a]	16 [a]	30 [a,b,c]	46 [a,b,c]	26 [a]	45 [a,b,c]	47 [a,b,c]
	(0)	(2)	(2)	(2)	(1)	(5)	(3)	(4)	(9)
Poorly differentiated, hepatocellular carcinoma	0	0	1	0	5 [a,b,c]	5 [a,c]	2	9 [a,b,c]	9 [a,b,c]
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(2)
Number of animals died of liver tumors	0	0	0	0	0	2	1	4	4

Symbols *a*, *b* and *c*: significantly different from the untreated control group, the each oral-alone group and each inhalation-alone group with matching concentrations, respectively, at $p < 0.05$ by chi-square test.

Parenthesized values indicate number of male rats dead and found in a moribund state, bearing hepatocellular tumors on the basis of histopathological examination.

Number of animals died of liver tumors was based on the primary cause of deaths diagnosed on the basis of macroscopic and microscopic findings.