Chemicals

Chloroform (purity greater than 99.0%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For the present study, chloroform vapor at a concentration of 100 ppm (v/v) was generated by bubbling clean air through liquid chloroform.

Animals

Male Crl:CD (SD) rats (SPF) 9 weeks of age were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Their body weights ranged from 341 to 368 g. Experiments were initiated after a one-week acclimation period. The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and the present study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC).

Study design

The performance of the whole-body inhalation exposure system was analyzed by the following 3 studies.

1. A performance study of the system without rats (study 1). The performance of the system without rats was analyzed by measuring chloroform vapor concentration and flow rate over the course of a single exposure day.

2. Confirmation of the performance of the system with rats (study 2). System performance with rats was analyzed by measuring chloroform vapor concentration and flow rate in the whole-body inhalation apparatus housing 6 rats over the course of a single exposure day. In addition, leakage of chloroform vapor from the tail-holder assembly of each animal-chamber was examined.

3. Use the system to analyze the concentration chloroform in the blood of test animals during the exposure to chloroform vapor (study 3). Six rats was were exposed to chloroform vapor (100 ppm) for 6 hr, and blood collected at 1, 30, 60, 120, 180, 240, 300 and 360 min from the start of exposure.

Analysis of chloroform vapor concentration in supply-header, animal-chamber and exhaust-header

Air in the supply-header, animal-chambers and exhaust-header was sampled using a RT731 (GL Sciences, Tokyo, Japan) every 15 min. Air at the rat tail outlet was sampled by a gas-tight syringe every 15 min. Chloroform vapor concentration was assessed by gas chromatography (GC) using Agilent Technologies 6890 (Agilent Technologies, Santa Clara, CA, USA) gas chromatography system (column, J&W DB-1 0.53 mmϕ × 5 m; oven temperature, 40°C; injection and detection temperature, 200°C; detector, FID; carrier gas, helium at 7.5 ml/min).

Rats blood sampling and treatment

Blood was collected from the tail vein of each rat during the inhalation exposure period at 1, 30, 60, 120, 180, 240, 300 and 360 min from the start of exposure. 0.1 ml blood samples were collected into 10-ml headspace sampler (HS)-vials and 0.1 ml of distilled water was added to each sample and the vials sealed immediately with an aluminum crimp cup.

Analysis of blood concentration

Blood samples were analyzed by HS-GC/mass spectrometry (MS) using Agilent Technologies 7694 (Agilent Technologies) headspace sampler (oven temperature, 80°C; loop temperature, 130°C; vial equilibration time, 10 min) and Agilent Technologies 5973N (Agilent Technologies) gas chromatography-mass spectrometry system (column, J&W DB-1 0.25 mmϕ × 60 m; oven temperature, 80°C; ion source temperature, 230°C; carrier gas, helium at 1 ml/min; ionization, EI; ionization voltage, 70 eV; fragment peak, 83 m/z).

RESULTS AND DISCUSSION

In the present study, a whole-body inhalation exposure system was designed to enable blood collection from rats during the period of exposure to a VOC; chloroform was used as a model VOC.

A performance study of the system without rats (study 1)

Table 1 (study 1) shows the chloroform vapor concentration at the sampling ports of the supply-header, animal-chamber and exhaust-header. The average concentrations in the supply-header, animal-chamber and exhaust-header over the course of a 6 hr exposure period were in the range of 100.0 to 101.5 ppm with coefficients of variation (C.V.) being less than 1.87%. 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, demonstrating good performance of the system.

Confirmation of system performance with rats (study 2)

Table 1 (study 2) shows the chloroform vapor concentration at the sampling ports of the supply-header, animal-chamber and exhaust-header. The average concentrations in the supply-header, animal-chamber and exhaust-head-

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.

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

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.

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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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.

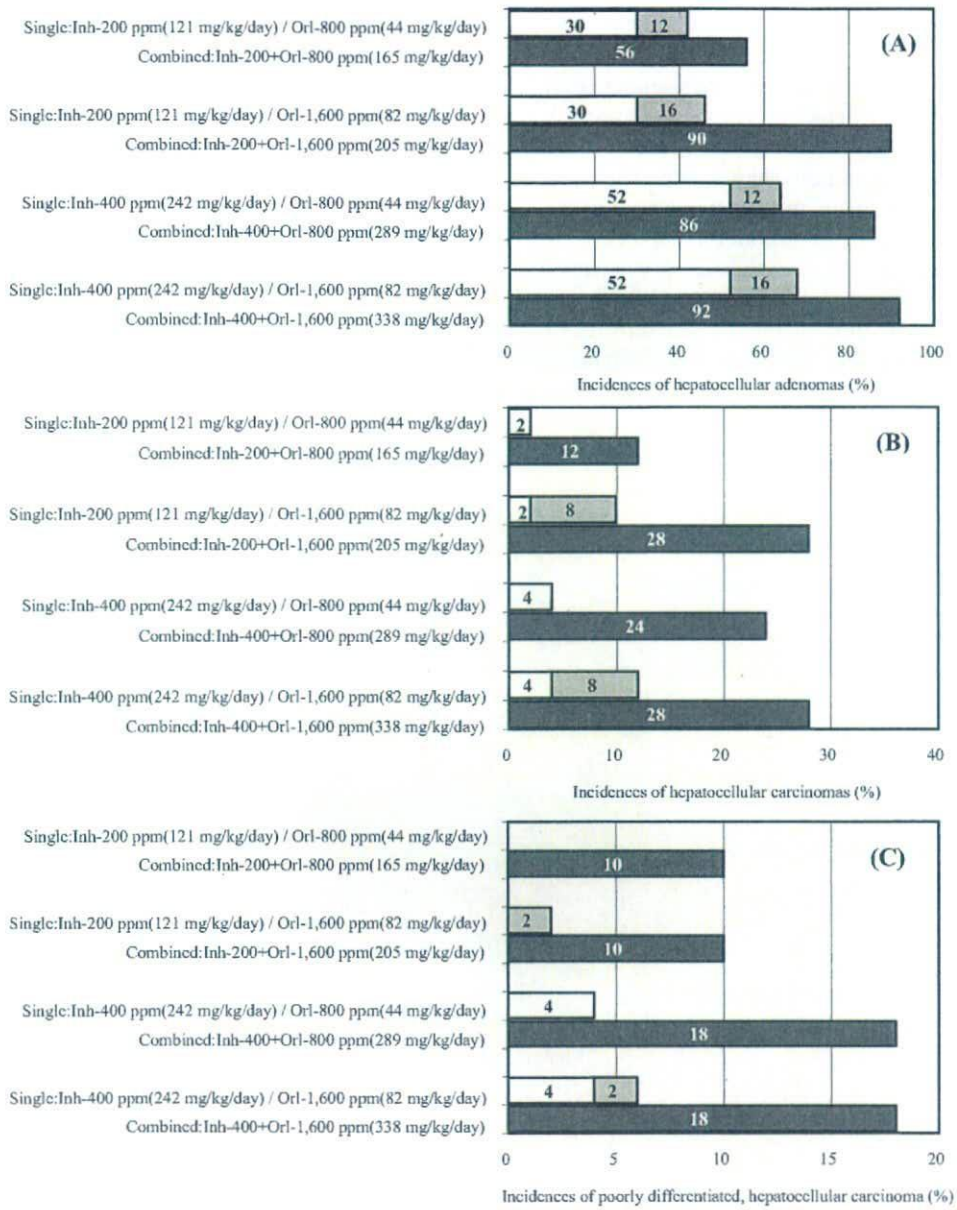

Fig. 1. Comparison of incidences of hepatocellular tumors ((A): hepatocellular adenomas, (B): hepatocellular carcinomas and (C): poorly differentiated hepatocellular carcinoma) in the combined-exposure groups (solid bars) with sum of the compornent incidences of the hepatocellular tumor in the single-route exposure groups through inhalation (open bars) and ingestion (shaded bars). The values in parenthesis indicate the total estimated amount of DMF uptake (mg/kg/day). The values in the bars represent the incidences of (A) hepatocellular adenomas, (B) hepatocellular carcinomas and (C) poorly differentiated, hepatocellular carcinoma.

were more remarkable than those of hepatocellular adenomas that are benign.

DMF-induced hepatocellular carcinomas can be classified into two different types according to the histopathological characteristics described by Senoh *et al.* (2004). The first type of hepatocellular carcinomas is prima-

rily composed of thick trabeculae of hepatocytes with abundant cytoplasm and round nuclei, which are similar to normal hepatocytes in histological appearance (Fig. 2A). The second type of hepatocellular carcinomas is composed of extremely thick trabeculae of hepatocytes with little cytoplasm and spindle-shaped hyperchromatic nuclei (Fig. 2B). The second type is considered to be more atypical and poorly differentiated, hepatocellular carcinomas, since the appearance of the second type deviates to a greater extent from that of normal hepatocytes than does the first type. Morphological characteristics of the second type became more malignant along the tumor-developmental sequence than those of the first type. The first type of hepatocellular carcinomas was found in all DMF-treated groups including the oral-alone, inhalation-alone and combined-exposure groups. The second type of hepatocellular carcinomas found in single-route exposure groups occurred at low incidences: 2/50 cases (4%) for the Inh-400+Orl-0 ppm group and 1/50 case (2%) for the Inh-0+Orl-1,600 ppm group. However, the combined exposures were found to markedly increase the incidence of the second type of hepatocellular carcinomas: 5/50 cases (10%) for the Inh-200+Orl-800 ppm, 5/50 cases (10%) for the Inh-200+Orl-1,600 ppm, 9/50 cases (18%) for the Inh-400+Orl-800 ppm, and 9/50 cases (18%) for the Inh-400+Orl-1,600 ppm. Notably, the inci-

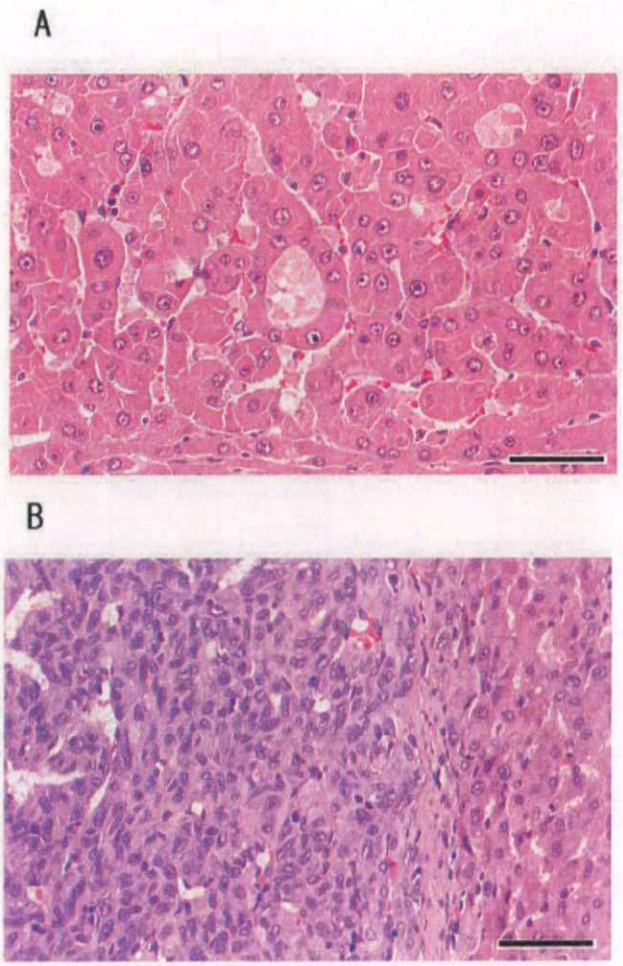

Fig. 2. (A): Hepatocellular carcinomas composed of thick trabeculae of hepatocytes with abundant cytoplasm and round nuclei in a male rat in the Inh-0+Orl-1,600 ppm group. (B): Poorly differentiated, hepatocellular carcinomas composed of extremely thick trabeculae of hepatocytes with little cytoplasm and spindle-shaped hyperchromatic nuclei in a male rat in the Inh-400+Orl-1,600 ppm group. H & E stain. Bar indicates 100 μm.

dences of the second type of hepatocellular carcinoma in the four combined-exposure groups were markedly greater than the sum of the two incidences of the second type of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1C). Therefore, the combined inhalation and oral exposures to DMF were found to markedly enhance not only the incidences of hepatocellular adenomas and carcinomas but also their tumor malignancy that might progress from hepatocellular adenomas through carcinomas to poorly differentiated, hepatocellular carcinomas.

It was noteworthy that the DMF-induced hepatocellular carcinoma did not metastasize to any other organs.

DISCUSSION

IARC's overall evaluation that DMF is not classifiable as to its carcinogenicity to humans (Group 3; IARC, 1999) was partly based on experimental evidence indicating lack of carcinogenicity after 2-year inhalation exposure of Crl:CD rats and CD-1 mice to DMF at an inhalation concentration of 25, 100 or 400 ppm (Malley *et al.*, 1994). More recently, however, our carcinogenicity study (Senoh *et al.*, 2004) has demonstrated that 2-year inhalation exposure to DMF vapor at 200, 400 and 800 ppm produces hepatocellular adenomas and carcinomas in F344 rats and BDF_1 mice of both sexes, although the exposure to 200 and 400 ppm primarily elicited benign hepatocellular adenomas. Senoh *et al.* (2004) suggested that the difference in carcinogenicity between Senoh *et al.*'s study and Malley *et al.*'s study was attributed to the strain of rats used. The present study confirms our previous findings, and extends those to the induction of hepatocellular tumors by oral administration of DMF in drinking water. Notably, the present study demonstrated that the combined inhalation and oral exposures of male F344 rats to DMF for 104 weeks markedly increase the incidences of hepatocellular tumors and their malignancy, as compared with the single-route exposures through inhalation and ingestion.

The extent to which the combined exposures markedly enhance the incidences of hepatocellular tumors is characterized by the hepatocarcinogenic effect being greater than the sum of the two hepatocarcinogenic effects of the single-route exposures through inhalation and ingestion. The "greater than additive" effect of the combined exposures on hepatocellular tumors tended to be accompanied by an increase in the malignancy of hepatocellular tumors. That is, the incidences of benign hepatocellular adenomas in the four combined-exposure groups were increased only by 1.3- to 2.0-fold over the sum of the two incidences of hepatocellular adenomas in the single-route exposure groups through inhalation and ingestion (Fig. 1A). The incidences of hepatocellular carcinomas in the four combined-exposure groups were increased by 2.3- to 6.0-fold over the sum of the two incidences of hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1B). Furthermore, the combined exposures enhanced by much greater folds the incidences of poorly differentiated, hepatocellular carcinomas, which are more malignant than the commonly observed hepatocellular carcinoma, over the sum of the two incidences of poorly differentiated, hepatocellular carcinomas in the single-route exposure groups through inhalation and ingestion (Fig. 1C). Therefore, the present findings suggest that the combined inhalation and oral exposures to DMF enhances the incidence of malignant hepatocellular tumors in a greater than additive manner, when we defined the additivity of carcinogenic responses as the same concept of "response additivity" applied to the effects of chemical mixture by the U.S. EPA (2000).

The characteristic relationship of total doses versus carcinogenic responses expressed as the incidences of hepatocellular tumors can be found in the two combined-exposure groups of Inh-200+Orl-800 ppm and Inh-200+Orl-1,600 ppm, both of which had total estimated DMF uptakes of 165 and 205 mg/kg/day, respectively. It is noteworthy that the incidences of hepatocellular carcinomas were greater in these two combined-exposure groups than in the Inh-400+Orl-0 ppm group having the total estimated DMF uptake of 242 mg/kg/day, although the former two combined-exposure groups had lesser uptakes than the latter single-route exposure group did. This finding also indicates that the hepatocarcinogenic effect induced by the combined inhalation and oral exposures would be enhanced in a greater than additive manner as expected from sum of the two effects induced by the single-route exposure through inhalation and ingestion.

The present carcinogenic effect of the combined exposures is in sharp contrast to that found in an inhalation study by Senoh *et al.* (2004) who reported 13/50 cases of hepatocellular adenomas with null case of hepatocellular carcinomas following 2-year inhalation exposure of male F344 rats to 400 ppm DMF vapor, which corresponded to an estimated DMF uptake of 242 mg/kg/day. Comparison of the estimated DMF dose-carcinogenic response relationships between the present combined-exposure study and Senoh *et al.*'s inhalation study (2004) also indicates that the combined inhalation and oral exposures to DMF produce greater incidences of hepatocellular tumors with higher malignancy than the inhalation-alone exposure

does.

We have not yet obtained any experimental evidence to elucidate the markedly enhanced hepatocarcinogenic effect of the combined inhalation and oral exposures to DMF. However, a clue to understanding the intriguing carcinogenic responses by the combined exposures can be seen in the finding in our previous study (Ohbayashi *et al.*, 2008) that the combined inhalation and oral exposures of male rats to DMF for 4 weeks markedly enhanced the proliferation index expressed as the percentage of PCNA-positive hepatocytes in a greater than additive manner as compared with the sum of the two proliferation indices by the single-route exposures through inhalation and ingestion. Since a broad range of *in vitro* and *in vivo* genotoxicity assays showed negative genotoxicity responses to DMF (IARC, 1999), a nongenotoxic-cytotoxic-proliferative mode of action can be hypothesized to operate for the DMF-induced hepatocarcinogenesis. This hypothesis suggests that hepatocellular death by the toxic insult of DMF and/or its active metabolites and the subsequently increased regenerative proliferation of hepatocytes play a crucial role in DMF-induced hepatocarcinogenesis (Butterworth *et al.*, 1992). Since both *N*-methylformamide (NMF), which was metabolized from DMF through *N*-(hydroxymethyl)-*N*-methylformamide and methyl isocyanate, which was possibly biotransformed from NMF were reported to be the most potent hepatotoxicants (Kestell *et al.*, 1987; Gescher, 1993; Mráz *et al.*, 1989, 1993), these two metabolites might cause severe hepatocellular damage that would result in enhanced cell proliferation in DMF-induced hepatocarcinogenesis. Further quantitative investigations such as physiological based pharmacokinetic modeling in consideration of the hepatic levels of DMF and its toxic metabolites are needed to examine greater than additive effects of the combined exposures to DMF on the hepatocellular tumors.

In conclusion, the present study demonstrated that the combined exposures of male rats to DMF at approximately similar dose levels each through inhalation and ingestion enhance induction of hepatocellular tumors and their malignancy in a greater than additive manner (i.e., possibly synergistic). In addition to the reported nongenotoxicity of DMF suggesting the presence of a threshold level for the tumor induction, sufficient consideration should be paid to delineation of the relationship between the dose levels given through the multiple routes and carcinogenic responses, in order to estimate quantitatively carcinogenic risks of humans exposed to an environmentally ubiquitous carcinogen such as DMF. Indeed, combined exposures to DMF at environmentally relevant levels is anticipated to occur for community residents living near a neighboring factory where DMF is used (Amster *et al.*, 1983) and/or public water contaminated with DMF, although a general population may be exposed to DMF at far lower levels through inhalation and ingestion.

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Thirteen-Week Inhalation Toxicity of 1,4-Dioxane in Rats

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Thirteen-week inhalation toxicity of 1,4-dioxane was examined by repeated inhalation exposure of male and female F344 rats to 0 (control), 100, 200, 400, 800, 1600, 3200, or 6400 ppm (v/v) 1,4-dioxane vapor for 6 h/day and 5 days/wk. All the 6400-ppm-exposed males and females died during the first week. Terminal body weight decreased, and relative weights of liver, kidney, and lung increased. AST increased in the 200 ppm- and 3200-ppm-exposed females, and ALT increased in the 3200-ppm-exposed males and females. Nuclear enlargement of nasal respiratory epithelial cells occurring in the 100-ppm-exposed males and females was the most sensitive, followed by the enlarged nuclei in the olfactory, tracheal, and bronchial epithelia. 1,4-Dioxane-induced liver lesions occurred at higher exposure concentrations than the nasal lesions did, and were characterized by single-cell necrosis and centrilobular swelling of hepatocytes in males and females. Glutathione *S*-transferase placental form (GST-P) positive liver foci were observed in the 1600-ppm-exposed females and 3200-ppm-exposed males and females, which are known as a preneoplastic lesion in rat hepatocarcinogenesis. Plasma levels of 1,4-dioxane increased linearly with an increase in the concentrations of exposure to 400 ppm and above. The enlarged nuclei in the nasal epithelia and the GST-P-positive liver foci were discussed in light of the possible development of nasal and hepatic tumors by long-term inhalation exposure to 1,4-dioxane. A lowest-observed-adverse-effect level (LOAEL) was determined at 100 ppm for the nasal endpoint in both male and female rats.

1,4-Dioxane is a highly flammable liquid, and has been widely used as a solvent for cellulose acetate, resins, oils, and waxes, and as a stabilizer in chlorinated solvents (Budavari et al., 1989; Lewis, 1993). The worldwide production volume of 1,4-dioxane was estimated to be 10000 tons in 1995 (EU Risk Assessment Report, 2002). The annual production of 1,4-dioxane in Japan was reported to amount to 4500 tons in 2004 (Chemical Daily, 2006). In the United States, release of 1,4-dioxane to atmosphere and surface water from manufacturing and processing facilities was estimated as 52 and 41 tons in 2004, respectively (Agency for Toxic Substances and Disease Registry [ATSDR], 2007). According to the Pollutant Release and Transfer Register Report from the Japan Ministry of the Environment (2007), 69 and 79 tons of 1,4-dioxane were released into atmosphere and public waters, respectively, from chemical industry in 2005. The National Institute for Occupational Safety and Health (NIOSH, 1977) reported that workplace air concentrations of 1,4-dioxane at 3 plants were 1.07, 0.9, and 6.5 ppm on average and the corresponding maximum values were 7.2, 2.0, and 23.6 ppm. Atmospheric concentrations of 1,4-dioxane at sampling points from various areas of Japan in 2000 were reported to range from 15 to 1200 ng/m^3 (0.3 ppb), while concentrations of 1,4-dioxane in public waters ranged from 0.08 to 160 μg/L (Japan Ministry of the Environment, 2002). Workers are at health risk when excessively exposed to 1,4-dioxane through inhalation and dermal contact in workplaces, while general populations are exposed to low levels of 1,4-dioxane in the urban ambient air through inhalation and in public waters through ingestion, inhalation, and dermal contact (DeRosa et al., 1996).

Excessive exposure of humans to 1,4-dioxane has been reported to cause deaths resulting from hepatic and renal failures in the acute and subacute phases (Johnstone, 1959; Rowe & Wolf, 1982). There have been no animal studies on subchronic and chronic inhalation toxicities of 1,4-dioxane, except for the

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Torkelson et al. study (1974) that found that repeated inhalation exposure of rats to 1,4-dioxane vapor at 111 ppm for 2 yr induced neither pathological changes nor tumors. The American Conference of Governmental Industrial Hygienists (ACGIH, 2001) and the German Research Foundation (DFG, 2003) established an occupational exposure limit (OEL) value of 20 ppm for 1,4-dioxane. The Japan Society for Occupational Health (JSOH, 1984) recommended an OEL value of 10 ppm for 1,4-dioxane, on the grounds that the OEL value is equivalent to one-tenth of the inhalation exposure level that did not induce any tumor in experimental animals. NIOSH (1977) recommended that occupational exposure to 1,4-dioxane be controlled so that employees are not exposed at airborne concentrations greater than 1 ppm, in the belief that 1,4-dioxane can be tumorigenic.

The present study was designed to better characterize the subchronic inhalation toxicity of 1,4-dioxane and to provide basic data on dose-response relationships for various endpoints for health risk assessment of humans exposed by inhalation to 1,4-dioxane vapor. In the present study, male and female F344 rats were exposed by inhalation to 1,4-dioxane vapor at 7 different concentrations or clean air for 6 h/day, 5 days/wk, for 13 wk. A potentially preneoplastic lesion of nuclear enlargement of epithelial cells in the nasal cavity and a preneoplastic lesion of glutathione *S*-transferase placental form (GST-P) positive liver foci were explored for detecting an early detectable stage participating in the possible development of nasal and hepatic tumors that would be induced after long-term inhalation exposure to 1,4-dioxane vapor. The OEL was discussed in light of the most sensitive nasal response to the inhaled 1,4-dioxane.

MATERIALS AND METHODS

The present study was conducted with reference to the Organization for Economic Cooperation and Development (OECD) Guideline for Testing of Chemicals 413 (OECD, 1981). The present study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC). The animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 1977).

Chemicals

1,4-Dioxane of reagent grade (greater than 99% pure) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The stock 1,4-dioxane was analyzed for purity and stability by gas chromatography–mass spectrometry (GC-MS) (Hitachi M-80B spectrometer, Hitachi, Ltd., Tokyo), gas chromatography (GC) (Hewlett Packard 5890, Agilent Technol., Santa Clara, CA), and infrared spectrometry (Hitachi 270-30, Hitachi, Ltd., Tokyo) before and after its use. Butylhydroxytoluene was detected in the 1,4-dioxane liquid by the GC-MS, and its concentration was quantitated as 1.3 ppm (w/w) by the GC. No gas chromatographic peak corresponding to butylhydroxytoluene was found in the air samples collected from the inhalation exposure chamber.

Animals

Four-week-old F344/DuCrj rats of both sexes were obtained from Charles River Japan, Inc. (Kanagawa, Japan). The animals were quarantined and acclimated for 2 wk, and then divided by stratified randomization into 8 body-weight-matched groups, each comprising 10 rats of both sexes. Inhalation exposure chambers were installed in the barrier system animal room where a temperature of 21 ± 2°C and a relative humidity of 60 ± 10% with 15–17 air changes/h were maintained. The animals were housed individually in stainless steel wire hanging cages (150 mm [W] × 216 mm [D] × 176 mm [H]) in the inhalation exposure chambers. The exposure chambers were maintained at a temperature of 20 to 24°C and a relative humidity of 30 to 70% with 12 air changes/h throughout the 13-wk exposure period. A 12-h light/dark cycle was automatically controlled. All the animals had free access to sterilized commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) and sterilized drinking water supplied by an automatic watering system.

Inhalation Exposure to 1,4-Dioxane Vapor

The animals were exposed to 1,4-dioxane vapor at a target concentration of 100, 200, 400, 800, 1600, 3200, or 6400 ppm for 6 h/day, 5 days/wk, for 13 wk. Groups of 10 rats of both sexes were exposed to clean air for 13 wk under the same conditions, and served as controls. Airflow containing 1,4-dioxane vapor at the target concentration was prepared by a vaporization technique. The saturated vapor–air mixture was generated by bubbling clean air through the 1,4-dioxane liquid in a temperature-regulated glass flask (30°C), and by cooling it through a thermostatted condenser at 20°C. The airflow containing the saturated vapor was diluted with clean air, and then warmed to 30°C in a thermostatted circulator that served to stabilize the vapor concentration by complete gasification of 1,4-dioxane. The flow rate of the vapor–air mixture was regulated with a flow meter, further diluted with humidity- and temperature-controlled clean air in a spiraling line mixer, and then supplied to an inhalation exposure chamber. Eight inhalation exposure chambers of 1060 L in volume were used in the present study. Each exposure chamber accommodated 20 individual cages for 10 males and 10 females. Chamber concentrations of 1,4-dioxane were monitored every 15 min with a gas chromatograph (GC-14B, Shimadzu Corp., Kyoto, Japan), equipped with a hydrogen flame ionization detector and a 1.5-m Shimadzu SBS-120 packed column operated at a column temperature of 90°C and with a gas injection volume of 2 ml. The concentrations were maintained constant at 100.2 ± 2.1 (mean ± SD), 200.7 ± 4.1, 403.9 ± 7.5, 799.8 ± 12.1, 1596.4 ± 27.2, 3198.4 ± 48.9 ppm throughout the 13-wk exposure period and 6409.5 ± 76.3 ppm for the first 1-wk surviving period. Accuracy and precision of the actual concentrations of 1,4-dioxane in the exposure chamber were kept by periodic injection of the certified standard 1,4-dioxane gas (Takachiho Co., Ltd., Tokyo) into the gas chromatograph for the calibration curve of 1,4-dioxane.

Clinical Observations and Analysis, and Pathological Examinations

The animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured once per week. All animals underwent complete necropsy. The organs were removed, weighed, and examined for macroscopic lesions. For hematology and blood biochemistry, the surviving animals were bled from the abdominal aorta under diethyl ether anesthesia after overnight fasting at the end of the 13-wk exposure period. Hematological parameters were measured with an automatic blood cell analyzer (TECHNICON H-1, Technicon Instruments Corp., New York) and an automatic blood cell differential analyzer (Hitachi 8200, Hitachi, Ltd., Ibaraki, Japan). Blood biochemical parameters were measured with an automatic analyzer (Hitachi 705) and a flame photometer (Hitachi 750, Hitachi, Ltd., Ibaraki, Japan). Urinary parameters were measured in the last week of the 13-wk exposure period with Ames reagent strips (Multistix, Bayer Corp., New York). All the organs and tissues designated in the OECD test guideline (OECD, 1981) and the entire respiratory tract including the nasal cavity, pharynx, larynx, trachea, and bronchus were examined for histopathology. The tissues were fixed in 10% neutral buffered formalin, and embedded in paraffin. The nasal cavity was decalcified in a formic acid–formalin solution prior to trimming, and was transversely trimmed at three levels according to the procedure described in our previous paper (Nagano et al., 1997), i.e., at the level of the posterior edge of the upper incisor teeth (Level 1), at the incisive papilla (Level 2), and at the level of the anterior edge of the upper molar teeth (Level 3). Tissue sections of 5 μm in thickness were prepared, and stained with hematoxylin and eosin (H&E). Additionally, the livers of all the ten 800-, 1600-, and 3200-ppm-exposed rats of both sexes and ten control rats of both sexes were sectioned for further examination of the enzyme-altered liver foci by immunohistochemical staining with anti-GST-P (Sato et al., 1984; Tatematsu et al., 1985; Ito et al., 1988), using EnVision+ (EV+, Dako, Copenhagen, Denmark) of the two-layer dextran polymer visualization system (Vyberg & Nielsen, 1998). Polyclonal antibody of GST-P (Anti-GST-P) was obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). Focal populations each having 50 or more hepatocytes homogeneously stained brown were defined as the GST-P-positive liver foci in the present study.

Determination of 1,4-Dioxane in Blood

Three rats of each sex per group except for the control were used to collect blood about 1 h after termination of day 3 exposure in wk 12 of the 13-wk exposure period. A 0.5-ml blood sample was collected from the tail vein into a heparinized tube and centrifuged at 3000 rpm for 5 min at 5°C. Then 100 μl plasma was diluted with 15 ml distilled water in a glass vial. 1,4-Dioxane was quantitatively determined with the headspace sampler gas chromatography–mass spectrometry (HS/GC-MS) (HS, Hewlett Packard 7694; GC-MS, Hewlett Packard 5989, Agilent Technol., Santa Clara, CA).

Statistics and Data Analysis

Body weight, food consumption, organ weight, and hematological and blood biochemical parameters were analyzed by Dunnett's test as described previously (Aiso et al., 2005). Histopathological findings and urinary parameters were analyzed by chi-square test. A two-sided analysis with p values of .05 and .01 was performed to determine statistical significance.

A no-observed-adverse-effect level (NOAEL) or a lowest-observed- adverse-effect level (LOAEL) was determined according to the WHO definition (International Programme on Chemical Safety [IPCS], 1994).

RESULTS

Survival, and Body Weights and Organ Weights

All the 6400-ppm-exposed rats of both sexes died during wk 1 of the 13-wk exposure period. The histopathological examination revealed that their deaths were primarily caused by renal failure, because all the 6400-ppm-exposed animals suffered from marked necrosis in the renal tubules. Moreover, lung congestion was observed in the 6400-ppm-exposed males and females (data not shown). All the other groups survived to the end of the 13-wk exposure period. In the surviving animals, no abnormal clinical sign was found in any 1,4-dioxane-dosed rats of either sex throughout the 13-wk exposure period. Terminal body weight significantly decreased in the 200-ppm- and 3200-ppm-exposed males and in the females exposed to 200 ppm, 800 ppm, and above (Table 1). Relative liver weight significantly increased in the males and females exposed to 800 ppm and above (Table 1). Relative kidney weight significantly increased in the 3200-ppm-exposed males and in the females exposed to 800 ppm and above. Relative lung weight increased in the males exposed to 200 ppm and 1600 ppm and above and in the females exposed to 200 ppm and above.

Hematology, Blood Biochemistry, and Urinalysis

Some erythrocyte parameters were increased in the 3200-ppm-exposed males and females with slight but statistical significance (Table 2). A slight but statistically significant increase was found in both aspartate aminotransferase (AST) in the 200-ppm- and 3200-ppm-exposed females and alanine aminotransferase (ALT) in the 3200-ppm-exposed males and females. The exposure to 3200 ppm decreased blood levels of glucose and triglyceride only in the males, but not in the females. Urinary protein slightly decreased in the males exposed to 3200 ppm (data not shown).

Histopathology

Repeated inhalation exposure to 1,4-dioxane vapor for 13 wk affected the upper and lower respiratory tracts and liver in both males and females and kidneys in females (Tables 3 and 4). The most sensitive lesion occurred in the nasal cavity,

TABLE 1

Terminal body weights and relative organ weights of the male and female rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

Group (ppm)	Control	100	200	400	800	1600	3200
Male							
No. of animals examined	10	10	10	10	10	10	10
Body weight (g)	323 ± 14	323 ± 14	304 ± 11*	311 ± 19	317 ± 12	312 ± 14	301 ± 11**
Liver (%)	2.610 ± 0.069	2.697 ± 0.092	2.613 ± 0.084	2.666 ± 0.080	2.726 ± 0.082*	2.737 ± 0.077**	2.939 ± 0.101**
Kidneys (%)	0.589 ± 0.016	0.596 ± 0.021	0.612 ± 0.013	0.601 ± 0.020	0.610 ± 0.015	0.606 ± 0.021	0.647 ± 0.026**
Lungs (%)	0.310 ± 0.011	0.312 ± 0.007	0.325 ± 0.008*	0.320 ± 0.009	0.321 ± 0.011	0.333 ± 0.009**	0.346 ± 0.017**
Female							
No. of animals examined	10	10	10	10	10	10	10
Body weight (g)	187 ± 5	195 ± 8	174 ± 10**	180 ± 5	175 ± 6**	173 ± 8**	168 ± 4**
Liver (%)	2.353 ± 0.081	2.338 ± 0.092	2.395 ± 0.092	2.408 ± 0.066	2.513 ± 0.076**	2.630 ± 0.139**	2.828 ± 0.144**
Kidneys (%)	0.647 ± 0.014	0.631 ± 0.019	0.668 ± 0.012	0.662 ± 0.024	0.679 ± 0.018**	0.705 ± 0.028**	0.749 ± 0.024**
Lungs (%)	0.402 ± 0.013	0.402 ± 0.015	0.435 ± 0.018**	0.429 ± 0.029*	0.430 ± 0.013**	0.454 ± 0.018**	0.457 ± 0.016**

Note. Values are represented as mean ± SD. Significant difference indicated by $^{*}p \leq .05$ and $^{**}p \leq .01$ by Dunnett's test.
%: Relative organ weight.
Data of the 6400-ppm-exposed groups are not presented here, because all of them died at the first wk of the 13-wk exposure period.

TABLE 2

Hematology and blood biochemistry of the male and female rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

Group (ppm)	Control	100	200	400	800	1600	3200
Male							
No. of animals examined	10	10	10	10	10	10	10
Red blood cell ($10^6/\mu L$)	9.55 ± 0.17	9.53 ± 0.24	9.54 ± 0.18	9.59 ± 0.26	9.55 ± 0.18	9.58 ± 0.14	9.57 ± 0.37
Hemoglobin (g/dL)	16.0 ± 0.2	16.1 ± 0.4	15.9 ± 0.2	16.1 ± 0.3	16.0 ± 0.3	16.2 ± 0.3	16.4 ± 0.4*
Hematocrit (%)	46.2 ± 1.2	46.3 ± 1.3	46.3 ± 0.9	46.3 ± 1.4	46.3 ± 1.1	46.8 ± 0.9	47.3 ± 1.7
MCV (fL)	48.4 ± 0.7	48.6 ± 0.7	48.6 ± 0.4	48.3 ± 0.4	48.5 ± 0.6	48.9 ± 0.6	49.4 ± 0.5**
AST (IU/L)	73 ± 8	75 ± 14	73 ± 10	72 ± 5	72 ± 3	70 ± 4	73 ± 4
ALT (IU/L)	27 ± 3	27 ± 4	27 ± 4	28 ± 1	27 ± 2	27 ± 2	30 ± 2*
Glucose (mg/dL)	197 ± 17	206 ± 13	192 ± 9	190 ± 12	187 ± 15	184 ± 12	170 ± 11**
Triglyceride (mg/dL)	125 ± 17	148 ± 37	118 ± 33	131 ± 30	113 ± 27	106 ± 24	87 ± 22*
Female							
No. of animals examined	10	9[a]	9[a]	9[a]	10	9[a]	10
Red blood cell ($10^6/\mu L$)	8.77 ± 0.23	8.69 ± 0.21	8.73 ± 0.25	8.88 ± 0.21	8.68 ± 0.69	8.86 ± 0.16	9.15 ± 0.12**
Hemoglobin (g/dL)	16.2 ± 0.3	16.0 ± 0.3	16.3 ± 0.4	16.2 ± 0.4	16.2 ± 0.6	16.3 ± 0.2	16.6 ± 0.2*
Hematocrit (%)	46.0 ± 1.5	45.5 ± 1.2	45.8 ± 1.7	46.5 ± 1.5	45.4 ± 3.6	46.2 ± 0.7	47.5 ± 0.6*
MCV (fL)	52.5 ± 0.7	52.3 ± 0.7	52.4 ± 0.7	52.4 ± 0.8	52.3 ± 0.6	52.1 ± 0.5	52.0 ± 0.7
AST (IU/L)	64 ± 6	65 ± 3	74 ± 14*	69 ± 5	68 ± 6	70 ± 5	76 ± 5**
ALT (IU/L)	23 ± 3	21 ± 2	26 ± 10	25 ± 3	24 ± 4	25 ± 3	30 ± 3**
Glucose (mg/dL)	143 ± 18	144 ± 18	137 ± 9	140 ± 15	141 ± 15	139 ± 11	139 ± 18
Triglyceride (mg/dL)	45 ± 5	48 ± 6	42 ± 4	47 ± 8	42 ± 6	39 ± 7	42 ± 7

Note. Values are represented as mean ± SD. Significant difference indicated by $^{*}p \leq .05$ and $^{**}p \leq .01$ by Dunnett's test.
MCV: Mean corpuscular volume. AST : Aspartate aminotransferase. ALT: Alanine aminotransferase.
[a]The measurement could not be carried out for 1animal because of shortage of blood volume.
Data of the 6400-ppm-exposed groups are not presented here, because all of them died at the first wk of the 13-wk exposure period.

TABLE 3
Incidences and severities of selected lesions in the male rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

	Male						
Group (ppm)	Control	100	200	400	800	1600	3200
No. of animals examined	10	10	10	10	10	10	10
Nasal cavity							
Nuclear enlargement: respiratory epithelium	0	7** (1+:7)	9** (1+:9)	7** (1+:7)	10** (1+:10)	10** (2+:10)	10** (2+:10)
Nuclear enlargement: olfactory epithelium	0	0	5* (1+:5)	10** (1+:10)	10** (1+:10)	10** (2+:10)	10** (2+:10)
Vacuolic change: olfactory epithelium	0	1 (1+:1)	3 (1+:3)	6* (1+:6)	10** (1+:10)	10** (1+:10)	9** (1+:9)
Trachea							
Nuclear enlargement: epithelium	0	0	0	0	1 (1+:1)	10** (1+:10)	10** (1+:10)
Bronchus							
Nuclear enlargement: bronchial epithelium	0	0	0	0	0	9** (1+:9)	10** (1+:10)
Vacuolic change: bronchial epithelium	0	0	0	0	4 (1+:4)	6* (1+:6)	6* (1+:6)
Liver							
Necrosis: single cells	0	0	0	0	0	1 (1+:1)	8** (1+:8)
Swelling: centrilobular	0	0	0	0	0	1	10** (1+:10)

Note. The number of the animals bearing the lesion in each exposed or control group are shown in the upper column. The parenthesized values indicate the number of the animals bearing the lesion with each of 4 different grades of severity ie., 1+: slight, 2+: moderate, 3+: marked, 4+: severe.

Significant difference indicated by* $p \leq .05$, ** $p \leq .01$ by chi square test.

Data of the 6400-ppm-exposed groups are not presented here, because all of them died at the first wk of the 13-wk exposure period.

where the incidences and severities of nuclear enlargement in the respiratory epithelium were significantly increased dose-dependently in both males and females exposed to 100 ppm and above. The nuclear enlargement (Figure 1) was morphologically characterized by the appearance of the epithelial cells having round to oval or elongated nuclei that were at least four times as large in diameter as the normal nuclei of respiratory epithelial cells. The enlarged nuclei of respiratory epithelial cells were

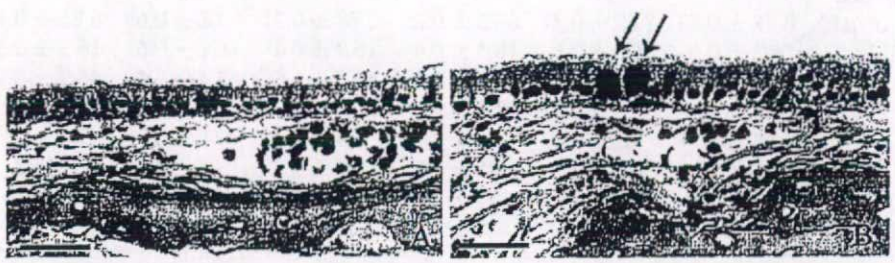

FIG. 1. (A) The normal respiratory epithelium in a male rat exposed to clean air for 13 wk. (B) Nuclear enlargement (arrows) in the respiratory epithelium at Level 2 of nasal cavity in a male rat exposed to 3200 ppm 1,4-dioxane for 13 wk. H&E stain. Bars indicate 50 μm.

TABLE 4
Incidences and severities of selected lesions in the female rats exposed by inhalation to 1,4-dioxane vapor at 6 different concentrations or clean air for 13 wk

	Female						
Group (ppm)	Control	100	200	400	800	1600	3200
No. of animals examined	10	10	10	10	10	10	10
Nasal cavity							
Nuclear enlargement: respiratory epithelium	0	5* (1+:5)	9** (1+:9)	10** (1+:10)	10** (1+:10)	10** (2+:10)	10** (2+:10)
Nuclear enlargement: olfactory epithelium	0	2 (1+:2)	6* (1+:6)	10** (1+:9, 2+:1)	10** (1+:10)	10** (1+:7, 2+:3)	10** (2+:10)
Vacuolic change: olfactory epithelium	0	1 (1+:1)	2 (1+:2)	3 (1+:3)	7** (1+:7)	9** (1+:9)	10** (1+:10)
Atrophy: olfactory epithelium	0	0	2 (1+:2)	3 (1+:3)	5* (1+:5)	5* (1+:5)	4 (1+:4)
Trachea							
Nuclear enlargement: epithelium	0	0	0	0	2 (1+:2)	7** (1+:7)	10** (1+:10)
Bronchus							
Nuclear enlargement: bronchial epithelium	0	0	0	0	0	0	10** (1+:10)
Vacuolic change: bronchial epithelium	0	0	0	1 (1+:1)	1 (1+:1)	3 (1+:3)	4 (1+:4)
Liver							
Necrosis: single cells	0	0	0	0	0	0	3 (1+:3)
Swelling: centrilobular	0	0	0	0	0	1 (1+:1)	8** (1+:8)
Kidney							
Hydropic change: proximal tubule	0	0	0	0	0	0	6* (1+:6)

Note. The number of the animals bearing the lesion in each exposed or control group are shown in the upper column. The parenthesized values indicate the number of the animals bearing the lesion with each of 4 different grades of severity ie., 1+: slight, 2+: moderate, 3+: marked, 4+: severe.

Significant difference indicated by $^{*}p \leq .05$, $^{**}p \leq .01$ by chi square test.

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localized at Level 1 in the males exposed to 100 and 200 ppm and in the females exposed to 100 ppm, and at Levels 1 and 2 in the females exposed to 200 ppm, while the respiratory epithelial cells having the enlarged nuclei were extended over the entire respiratory region at Level 1 through 3 in both males and females exposed to 400 ppm and above. The enlarged nuclei of the sustentacular cells in the olfactory epithelium (Figure 2) were significantly increased in both males and females exposed to 200 ppm and above, and were distributed over almost the entire area of the olfactory region at Levels 2 and 3. The incidences and severities of nuclear enlargement in the 1,4-dioxane-exposed males and females tended to decrease along the passage of inspiratory airflow through the upper and lower respiratory tracts. The incidence of degenerative change in the olfactory sensory cells observed as the vacuolic change (Figure 2) was significantly increased in the males exposed to 400 ppm and above, while the females exposed to 800 ppm and above exhibited olfactory epithelial atrophy characterized by a decreased number of the olfactory sensory cells and vacuolic change. Vacuolic change in the bronchial epithelium also occurred with statistical significance in the males exposed to 1600 ppm and above.

The 1,4-dioxane-induced liver lesions were characterized by significant increases in the incidences of both single-cell necrosis and centrilobular swelling of hepatocytes in the 3200-ppm-exposed males, and in the incidence of centrilobular swelling of hepatocytes in the 3200-ppm-exposed females. In addition, the GST-P-positive liver foci (Figure 3) were observed in three 3200-ppm-exposed males, two 3200-ppm-exposed females, and