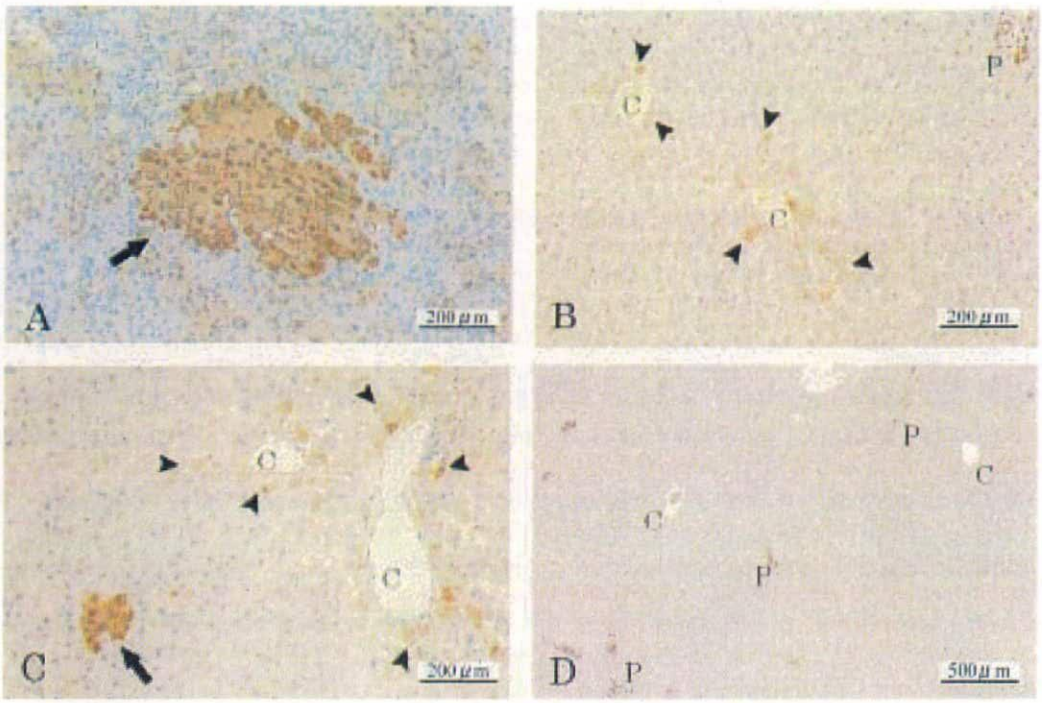

Fig. 1.
(A) A GST-P positive focus composed of 58 hepatocytes stained dark brown in a male rat administered 300 μg TBDD/kg body weight and sacrificed on Day 36. (B) A GST-P-positive area in a female rat administered 30 μg TBDD/kg body weight and sacrificed on Day 2. The severity grade was scored as 1+ (slight), less than 10% of total area occupied by the GST-P-positive area (arrowhead). (C) Both a GST-P-positive focus (thick arrow) composed of 10 hepatocytes and the GST-P-positive area (arrowhead) found in a male rat administered 300 μg TBDD/kg body weight and sacrificed on Day 36 (higher magnification). The severity grade of the positive area was scored as 2+ (moderate), 10 to 40% of the total area occupied by the GST-P-positive area. (D) Neither the GST-P-positive foci nor the GST-P-positive area found in a male rat administered vehicle solution (control group) and sacrificed on Day 36 (lower magnification). Note that only the bile duct weakly stained with anti-GST-P antibody can be seen in the portal triad. Portal triad (P). Central vein (C). All the sections were stained with anti-GST-P antibody using EnVison+ of two-layer dextran visualizing system.

posed of 2 to 58 hepatocytes and the GST-P-positive area of hepatocytes in the centrilobular region. The GST-P-positive foci found in the 100 and 300 μg/kg TBDD/kg-dosed rats exhibited morphologically focal and clonal proliferation of the hepatocytes. However, the conventional staining with hematoxylin and eosin did not allow clear detection of such small, enzyme-altered hepatocellular foci due to monochromatically altered staining intensity, because the number of the GST-P-positive hepatocytes in the foci was too small to detect by hematoxylin and eosin staining. The GST-P-positive hepatocellular foci were found to closely resemble the reported morphological features[22, 23] of the small, GST-P-positive foci which were induced shortly after a single ip injection of a genotoxic hepatocarcinogen, diethylnitrosamine (DEN) to the partially hepatectomized or intact rat. The DEN-induced, small GST-P-positive foci was suggested as an indication of the initiation of chemically-induced hepatocarcinogenesis[22, 23], because of the clonal growth of the GST-P-positive foci of small size from the single GST-P-positive hepatocyte. In addition, a single ip injection of DEN followed by 6-wk oral administration of DEN in drinking water in the partially hepatectomized rat was reported to induce the fully developed (focus of >0.2 mm in diameter), preneoplastic, GST-P-positive foci[24]. Slow elimination of TBDD from the body at a half-life ($t_{1/2}$) ranging from 12 to 16 d after the single oral administration of 100 and 300 μg/kg body weight[15] can be taken to indicate that the liver is internally exposed to TBDD at time-averaged levels greater than 100 ng/g liver tissue for a time period of 36 d during which the single GST-P-positive hepatocytes grow focally and clonally to the small, GST-P-positive liver foci. Therefore, it can be inferred that a portion but not all of the small, GST-p-positive hepatocellular foci occurring shortly after a single administration of TBDD may undergo further focal and clonal proliferation and progress finally to hepatocellular carcinomas, provided that the repeated administration of TBDD to rats is prolonged up to 2 yr. Indeed, positive hepatocarcinogenicity has been demonstrated in the

Table 1. GST-P positive foci and GST-P-positive area in the TBDD-dosed rats of both sexes sacrificed on Days 2, 7 and 36 after the single administration

Days after administration		Dose of TBDD (μg/kg body weight)	No. of animals examined	GST-P-positive foci						GST-P positive area	
				No. of bearing animals	No. of foci (/cm²)	No. of hepatocytes in the focus[a]				No. of bearing animals	Severity grade[b]
						2–5	6–10	11–20	21–60		
Male	2	0	5	0	0	0	0	0	0	0	0
		10	5	0	0	0	0	0	0	0	0
		30	5	0	0	0	0	0	0	1	0.2
		100	5	0	0	0	0	0	0	2	0.4
		300	5	0	0	0	0	0	0	5	1.0
	7	0	5	0	0	0	0	0	0	0	0
		10	5	0	0	0	0	0	0	0	0
		30	5	0	0	0	0	0	0	1	0.2
		100	5	0	0	0	0	0	0	3	0.8
		300	5	0	0	0	0	0	0	5	1.2
	36	0	5	0	0	0	0	0	0	0	0
		10	5	0	0	0	0	0	0	0	0
		30	5	0	0	0	0	0	0	0	0
		100	5	2	0.30	0	1	1	0	5	1.0
		300	5	3	0.59	2	2	0	1	4	1.6
Female	2	0	5	0	0	0	0	0	0	0	0
		10	5	0	0	0	0	0	0	0	0
		30	5	0	0	0	0	0	0	5	1.0
		100	5	0	0	0	0	0	0	4	0.8
		300	5	0	0	0	0	0	0	4	1.0
	7	0	5	0	0	0	0	0	0	0	0
		10	5	0	0	0	0	0	0	1	0.2
		30	5	0	0	0	0	0	0	4	0.8
		100	5	0	0	0	0	0	0	4	1.3
		300	5	0	0	0	0	0	0	5	1.6
	36	0	5	0	0	0	0	0	0	0	0
		10	5	0	0	0	0	0	0	2	0.4
		30	5	0	0	0	0	0	0	3	0.6
		100	5	3	2.43	1	1	7	8	4	1.6
		300	2[c]	2	2.00	0	3	3	0	1	1.0

[a] Values indicate number of GST-P-positive hepatocytes in each of the foci.

[b] The values indicate the averaged severity grade for the GST-P-positive area in each group. The severity grade and its averaging were given in Materials and Methods.

[c] Three female rats died before the scheduled necropsy on Day 36.

female rats orally administered TCDD for 2 yr[16, 17].

TCDD has been reported to promote hepatocarcinogenicity according to the studies[18–21] on the two-stage model for hepatocarcinogenesis, since no convincing evidence that it covalently binds to DNA or that TCDD is a mutagen has been reported to date[9]. Maronpot *et al.*[18] showed that TCDD induces the preneoplastic, GST-P-positive foci in the rat initiated with DEN and subsequently administered TCDD by oral gavage at 125 ng/kg/d for 30 wk, whereas the oral administration of TCDD at 125 ng/kg/d alone for 30 wk to the saline-injected, non-initiated rat did not significantly increase the GST-P-positive foci. Pitot *et al.*[19] showed that biweekly sc injections of TCDD at 0.14 and 1.4 μg/kg for 28 wk in the DEN-initiated, previously partially hepatectomized rat induce the enzyme-altered hepatocellular foci, while incidences of those enzyme-altered foci were not increased after the repeated subcutaneous injections of TCDD alone in the non-initiated, partially hepatectomized rat. TCDD was classified as a potent tumor-promoting agent and a weak or non-initiator, based on stereologic quantification of those enzyme-altered liver foci for relative potency of

chemicals as initiating or promoting agents[20]. Chronic treatment of the DEN-initiated rat with TCDD biweekly administered sc at 1.4 μg/kg for 115 d was reported to significantly increase the number of GST-P-positive foci[21]. Therefore, these two-stage model studies for hepatocarcinogenesis[18–21], together with no convincing evidence on the covalent binding of TCDD with DNA or mutagenicity of TCDD[9], indicate that repeated administration of TCDD to rats promotes the liver tumors through formation of the GST-P-positive foci initiated by DEN. However, it remains unsolved as to whether TBDD acts as a genotoxic hepatocarcinogen like DEN or as a non-genotoxic hepatocarcinogen like TCDD. Further studies on the covalent binding of TBDD with DNA and *in vitro* and *in vivo* genotoxicity of TBDD will be needed to explore any causative factor for formation of the GST-P-positive liver foci.

It is of interest to note that both the number of GST-P-positive foci and the number of GST-P-positive hepatocytes in each of the foci were greater in the TBDD-dosed female rats than in the males given the same amount of TBDD, suggesting a gender difference in the TBDD-induced, enzyme-altered hepatocellular foci. This finding might extend the previously reported findings[11, 14, 15, 37] of the enhanced TBDD hepatotoxicity of female rats to higher susceptibility of female rats to the formation of GST-P-positive liver foci than males. Lucier *et al.*[38] demonstrated using the two-stage hepatocarcinogenesis model that the number of GST-P-positive foci were greater in intact female rats receiving a single ip injection of DEN followed by repeated oral administration of TCDD at a dose equivalent to 100 ng/kg/d for 30 wk than in ovariectomized rats receiving DEN and TCDD. As demonstrated by Lucier *et al.*'s study[38], possible involvement of ovarian hormones in higher susceptibility of female rats to the TBDD-induced, GST-P-positive liver foci than males is suggested. Indeed, a possibility of the enhanced hepatic levels of TBDD in females as a cause of the enhanced susceptibility of females to formation of the GST-P-positive liver foci can be ruled out, since in the previous study[15] no significant difference in hepatic levels of TBDD was found between the female rats dosed 100 and 300 μg/kg and the males dosed the same amounts of TBDD.

Another finding was the area occupied by the hepatocytes which were stained heterogeneously with anti-GST-P antibody and present predominantly in the centrilobular region. Notably, the stained hepatocytes appeared to be neither focally nor clonally proliferating without clearly distinguishable demarcation from surrounding normal hepatocytes. Characteristics of the GST-P-positive area such as its occurrence in the centrilobular region and at dose levels lower than 100 μg/kg and its persistent appearance on Day 2 through 36 can be in sharp contrast to those of the focally and clonally proliferating GST-P-positive hepatocellular foci that occurred only on Day 36 in the 100 and 300 μg/kg-administered rats. In the previous study[15], both the centrilobular hypertrophy of hepatocytes and the concomitantly increased CYP1A enzyme activities measured as hepatic AHH and EROD activities occurred persistently on Day 2 through 36 after a single oral administration of TBDD at all dose levels from 10 to 300 μg/kg. Thus, the centrilobular localization of the GST-P-positive area is compatible with the previous finding[15] of centrilobular hypertrophy of hepatocytes in the TBDD-dosed rat which concomitantly exhibited the increased CYP1A enzyme activities. Centrilobular localization of immunohistochemically stained CYP1A1 and CYP1A2 was also observed in the liver of the DEN-initiated, TCDD-dosed rat[39]. It is noteworthy that GST-P form is induced *in vitro* in rat liver parenchymal cells by polychlorinated biphenyl having TCDD-like coplanar structure[32, 33]. Besides, induction of GSTs and CYP1A enzymes *in vitro* in human hepatocyte cultures by TCDD[31] and centrilobular localization of GSTs and P450 enzymes in the phenobarbital-treated rat[40] have been demonstrated. Taken together with these reported findings[15, 31–33, 39, 40], the centrilobular-localized, GST-P-positive area of hepatocytes without appearance of focal or clonal proliferation in the TBDD-dosed rat might reflect induction of detoxifying Phase II GSTs involved in the glutathione conjugation process of the hydroxylated TBDD after possible hydroxylation of TBDD by Phase I CYP1A enzymes.

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Original Article

Thirteen-week oral toxicity of 1,4-dioxane in rats and mice

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ABSTRACT — Subchronic oral toxicity of 1,4-dioxane was examined by administering 1,4-dioxane in drinking water at 6 different concentrations of 0 (control), 640, 1,600, 4,000, 10,000 or 25,000 ppm (wt/wt) to F344 rats and BDF_1 mice of both sexes for 13 weeks. Food and water consumption and terminal body weight were decreased dose-dependently in rats and mice. A dose-dependent increase in the relative weights of kidney and lung was noted in rats and mice, while the relative liver weight was increased only in rats. Increases in plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and a decrease in plasma glucose were noted primarily in the rats and mice dosed 25,000 ppm. Histopathological examination revealed that 1,4-dioxane affected the upper and lower respiratory tracts, liver, kidneys and brain in rats, while only the former two organs were affected in mice. Nuclear enlargement occurred in the respiratory, olfactory, tracheal and bronchial epithelia of the 1,4-dioxane-dosed rats and mice. The 1,4-dioxane-induced hepatic lesions were characterized by centrilobular swelling and necrosis in rats and mice and by glutathione *S*-transferase placental form (GST-P)-positive altered hepatocellular foci in rats, which are known as preneoplastic lesions. A no-observed-adverse-effect-level (NOAEL) was determined at 640 ppm for both rats and mice, since the nuclear enlargement in the nasal respiratory epithelium and the centrilobular swelling of hepatocytes in rats and the nuclear enlargement in the bronchial epithelium in mice were observed at 1,600 ppm. The NOAEL value corresponded to the estimated 1,4-dioxane intake of 52 mg/kg/day in rats and 170 mg/kg/day in mice.

Key words: 1,4-Dioxane, Subchronic toxicity, Mouse, Rat, Liver, Nasal cavity

INTRODUCTION

1,4-Dioxane is a highly flammable liquid, and has been widely used as a solvent for various organic products, as a reaction medium solvent in chemical manufacture, and as a stabilizer in chlorinated organic solvents (Inoue *et al.*, 1983; Kumai *et al.*, 1983; Chemical Daily, 2006). The annual production of 1,4-dioxane in 2004 was reported to amount to 4,500 tons in Japan (Chemical Daily, 2006). According to the Pollutant Release and Transfer Register Report from the Japan Ministry of the Environment (2007), 95 and 79 tons of 1,4-dioxane were released annually into the atmosphere and public water in 2005, respectively, primarily from chemical industry. Atmospheric concentrations of 1,4-dioxane at sampling points from various areas of Japan in 2000 were reported to range from 15 to 1,200 ng/m^3, while concentrations of 1,4-dioxane in public waters ranged from 0.08 to 160 μg/l (Japan Ministry of the Environment, 2002).

Principal routes of human exposure to 1,4-dioxane from occupational and environmental sources are inhalation, ingestion and dermal contact (DeRosa *et al.*, 1996). Workers are at health risk to be excessively exposed to 1,4-dioxane in its manufacturing and using processes, while general populations are exposed to low levels of 1,4-dioxane in the urban air and in the 1,4-dioxane-contaminated public water. 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 and Wolf, 1982). Few animal studies on subchronic toxicity of 1,4-dioxane are available for health risk assessments, although several bioassay studies of carcinogenicity by long-term oral administration of 1,4-dioxane in drinking water to rats and mice have been reported (Argus *et al.*, 1965, 1973; Hoch-Ligeti *et al.*, 1970; Kociba *et al.*, 1974; National

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Cancer Institute, 1978). 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 the OEL value of 10 ppm for 1,4-dioxane, on the ground that the OEL value is equivalent to one tenth of the inhalation exposure level which did not induce any tumor in experimental animals. The National Institute for Occupational Safety and Health (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, on the belief that 1,4-dioxane can be tumorigenic. Some regulatory standards of 1,4-dioxane in drinking water (Japan Ministry of Health, Labour and Welfare, 2003; WHO, 2005) recommended that water be below 0.05 mg/l for 1,4-dioxane, based on a linearized multistage model using data of 1,4-dioxane-induced rodent carcinogenicity. The United States Environmental Protection Agency (U.S. EPA, 2007) estimated the lifetime cancer risk of 1 in 1,000,000 as 3μg/l for 1,4-dioxane in drinking water, based on the NCI data (NCI, 1978) on the responses of rat nasal tumors to 1,4-dioxane in drinking water.

The purpose of the present study was to better characterize the subchronic oral toxicity of 1,4-dioxane for health risk assessment, with the emphasis on dose-response relationships for various endpoints in the subchronic toxicity. In addition, the occurrence of glutathione *S*-transferase placental form (GST-P)-positive hepatocellular foci was also explored for detecting an early recognizable stage developing to liver tumors, since the liver foci stained positively with GST-P have been reported to allow the prediction of hepatocarcinogenicity in rats with high probability (Ito *et al.*, 2000). In the present study, F344 rats and BDF_1 mice of both sexes were orally administered 1,4-dioxane in drinking water at 6 different concentrations for 13 weeks with reference to the Organisation for the Economic Co-operation and Development (OECD) Guideline for Testing of Chemicals 408 "Subchronic Oral Toxicity - Rodent: 90-day Study" (OECD, 1981a).

MATERIALS AND METHODS

The present study was conducted in accordance with the OECD Good Laboratory Practice (OECD, 1981b) and 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 (NIH, 1977).

Chemicals

1,4-Dioxane (spectrometric grade, more than 99.0% purity) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The test substance was analyzed for purity and stability by infrared spectrometry (270-30 Infrared Spectrometer, Hitachi Ltd., Ibaraki, Japan) and gas chromatography (GC-9A, Shimadzu Co., Kyoto, Japan) before and after its use. Neither decomposition products nor impurities were detected in the 1,4-dioxane.

Preparation of drinking water containing 1,4-dioxane and its stability

1,4-Dioxane was dissolved in deionized water to a target concentration of 640, 1,600, 4,000, 10,000 or 25,000 ppm (wt/wt). The 1,4-dioxane-formulated drinking water was prepared twice a week, and administered to each animal with a sipper bottle made of glass during a 3- or 4-day period. The volume of the sipper bottle was 200 ml for rats and 35 ml for mice. The concentrations of 1,4-dioxane in drinking water were determined at the time of preparation with the gas chromatograph, and found to be 94.6-102.9% of the target concentration. Stability of 1,4-dioxane in the drinking water was examined 4 days after preparation of the 1,4-dioxane-formulated drinking water, using the gas chromatograph and found to be 94.2-101.1% of the initial concentrations for rats and 92.8-96.4% for mice.

Animals

Four-week-old F344/DuCrj rats (SPF) and Crj: BDF_1 mice (SPF) of both sexes were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were quarantined and acclimated for 2 weeks, and then divided by stratified randomization into 6 body weight-matched groups, each comprising 10 rats and 10 mice of both sexes. The animals were housed individually in stainless steel wire-mesh hanging cages (170W × 294D × 176H mm for rats and 112W × 212D × 120H mm for mice) under controlled environmental conditions (temperature of 24±1°C and a relative humidity of 55±5%, with 15-17 room air changes/hr). Fluorescent lighting was controlled automatically to provide a 12-hr light/dark cycle. All animals were given *ad libitum* diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and the assigned drinking water during the 13-week administration period, starting at the age of 6 weeks.

Experimental design, clinical observations and analysis, and pathologic examinations

Groups of 10 rats and 10 mice of each sex per group were given the drinking water containing 640, 1,600,

4,000, 10,000 or 25,000 ppm for 13 weeks. Groups of 10 rats and 10 mice of both sexes serving as respective controls were given only deionized water for 13 weeks. The animals were observed daily for clinical signs and mortality. Body weight and food consumption were recorded once a week. Daily water consumption was calculated by subtracting the weight of drinking water remaining after the 3- or 4-day administration from the initial weight of water in the bottle, and divided by the days spent for the administration. A daily intake of 1,4-dioxane was estimated as the concentration of 1,4-dioxane in the drinking water, multiplied by the volume of drinking water consumed on a daily basis, and divided by the body weight.

All animals underwent complete necropsy. The organs were removed, weighed and examined for macroscopic lesions at necropsy. Blood was collected for hematology and blood biochemistry under etherization at the end of the 13-week administration period, after overnight fasting. Hematological parameters were measured with an Automatic Blood Cell Analyzer (Coulter Counter SP, Coulter Electronics Inc., NY, USA) and Automatic Blood Cell Differential Analyzer (Hematrak 590, Geometric Data and SmithKline Co., NY, USA). Blood biochemical parameters were measured with an Automatic Analyzer Hitachi 705 and a Flame Analyzer Hitachi 750 (Hitachi Ltd., Ibaraki, Japan). Urinary parameters were examined with Ames Reagent Strips (Multistix for rats, Uro-labstix for mice, Miles Sankyo, Co., Tokyo, Japan). The tissues specified in the OECD test guideline (OECD, 1981a) and the entire respiratory tract including nasal cavity, pharynx and larynx of all the animals were examined for histopathology. For microscopic examination, the tissues were fixed in 10% neutral buffered formalin, and were embedded in paraffin. Tissue sections of 5 μm thick were prepared, and were stained with hematoxylin and eosin (H and E). The nasal cavity was decalcified in formic acid-formalin solution prior to trimming, and was transversely trimmed at three levels according to the procedure described in the 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).

Additionally, the livers of five 25,000 ppm-dosed and five vehicle-dosed rats of both sexes were sectioned for further examination of altered hepatocellular foci by immunohistochemical staining with antibody of glutathione *S*-transferase placental form (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 and Nielsen, 1998). Polyclonal anti-GST-P was obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). Focal populations having fifty or more homogeneously stained brown and agglomerated hepatocytes were defined as the GST-P-positive foci in the present study.

Statistics

Body weight, water consumption, food consumption, organ weight, and hematological and blood biochemical parameters were analyzed by Dunnett's test as described in detail previously (Aiso *et al.*, 2005). Histopathological findings and urinary parameters were analyzed by chi-square test. A two-sided analysis with p values of 0.05 and 0.01 was performed to determine statistical significance.

A no-observed-adverse-effect-level (NOAEL) was determined for both rats and mice, according to the WHO definition (IPCS, 1994).

RESULTS

Survival, clinical observations, food and water consumption, and chemical intake

A female rat and a male mouse, both of which were given 25,000 ppm, died during the 2nd week of the 13-week administration period. Histopathological examination revealed that the death of the female rat was causally related to renal failure because of marked hydropic degeneration of the proximal tubule, whereas the cause of death in the male mouse could not be confirmed. In the surviving animals, piloerection and colored fur in both male and female rats given 25,000 ppm, and piloerection in the male mice given 25,000 ppm were observed. Food consumption was significantly decreased in the male rats given 25,000 ppm and in the female rats given 10,000 ppm and 25,000 ppm (Table 1), while significantly decreased food consumption was observed only in the male mice given 25,000 ppm. Water consumption was decreased dose-dependently in both male and female rats given 4,000 ppm and above, and in both male and female mice given 10,000 ppm and 25,000 ppm (Table 1). The amounts of water consumed were reduced to 52% and 42% for the male and female rats given 25,000 ppm and to 33% and 43% for the male and female mice given 25,000 ppm, respectively, in comparison with those consumed by the control animals. Notably, 1,4-dioxane intake did not increase proportionally with a 2.5-fold increase in the 1,4-dioxane concentration in drinking water.

Macroscopic findings, and body and organ weights

No particular macroscopic lesion was found in any 1,4-dioxane-dosed rats or mice of either sex at terminal necropsy. Terminal body weight was significantly decreased in male rats given 10,000 ppm and 25,000 ppm and in female rats given 4,000 ppm and above, while the significantly decreased body weight was observed only in male mice given 25,000 ppm (Table 2).

Relative liver weight was significantly increased in male rats given 10,000 ppm and 25,000 ppm and in female rats given 1,600 ppm and above, whereas no increase in relative liver weight was recognized in any 1,4-dioxane-dosed mouse group (Table 2). Relative kidney weight was significantly increased in male rats given 4,000 ppm and above and in female rats given 1,600 ppm and above, while significantly increased kidney weight was noted in the mice of both sexes given 25,000 ppm. Relative lung weight was significantly increased in both male rats and male mice given 25,000 ppm, and in both female rats and female mice given 10,000 ppm and 25,000 ppm (Table 2).

Hematology, blood biochemistry and urinalysis

Red blood cell counts, hemoglobin and hematocrit were significantly increased in both male rats and male mice given 25,000 ppm, whereas those three erythrocyte parameters were not increased in any 1,4-dioxane-dosed female group of rats or mice (Table 3).

Both aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly increased in male rats, male mice and female mice given 25,000 ppm. In addition, AST was significantly increased in female rats given 25,000 ppm, and ALT was also significantly increased in female mice given 10,000 ppm. Notably, a significant decrease in the plasma level of glucose was observed in rats and mice of both sexes given 25,000 ppm, and in female mice given 10,000 ppm (Table

Table 1. Food consumption, water consumption and chemical intake of the rats and mice orally administered 1,4-dioxane in drinking water at 5 different concentrations or vehicle water for 13 weeks.

(A) Rats

Group (ppm)		Control	640	1600	4000	10000	25000
<Male>							
No. of animals examined		10	10	10	10	10	10
Food consumption	(g/day)	17.2 ± 0.7	17.7 ± 0.7	* 17.9 ± 0.2	17.1 ± 0.6	17.0 ± 0.4	15.8 ± 0.6 **
Water consumption	(g/day)	27.2 ± 7.4	23.1 ± 3.7	22.7 ± 2.7	18.9 ± 1.0 **	17.6 ± 0.8 **	14.2 ± 0.9 **
Chemical intake	(mg/kg/day)	-	52 ± 9	126 ± 16	274 ± 10	657 ± 30	1554 ± 91
<Female>							
No. of animals examined		10	10	10	10	10	9 [a]
Food consumption	(g/day)	12.4 ± 0.6	12.8 ± 0.6	12.4 ± 0.8	11.9 ± 0.6	11.3 ± 0.5 **	10.3 ± 1.7 **
Water consumption	(g/day)	22.6 ± 5.9	23.1 ± 7.3	20.1 ± 5.1	18.2 ± 7.9 **	12.1 ± 1.2 **	9.4 ± 0.4 **
Chemical intake	(mg/kg/day)	-	83 ± 27	185 ± 42	427 ± 161	756 ± 56	1614 ± 121

(B) Mice

Group (ppm)		Control	640	1600	4000	10000	25000
<Male>							
No. of animals examined		10	10	10	10	10	9 [a]
Food consumption	(g/day)	4.1 ± 0.2	4.1 ± 0.2	4.1 ± 0.2	4.1 ± 0.2	3.9 ± 0.2	3.4 ± 0.7 **
Water consumption	(g/day)	4.8 ± 1.7	4.1 ± 0.6	4.3 ± 1.0	4.3 ± 1.4	2.7 ± 0.5 **	1.6 ± 0.4 **
Chemical intake	(mg/kg/day)	-	86 ± 17	231 ± 66	585 ± 253	882 ± 147	1570 ± 139
<Female>							
No. of animals examined		10	10	10	10	10	10
Food consumption	(g/day)	4.1 ± 0.3	4.0 ± 0.2	4.0 ± 0.3	4.1 ± 0.2	4.0 ± 0.2	3.8 ± 0.2
Water consumption	(g/day)	5.4 ± 0.4	5.9 ± 1.7	5.4 ± 1.0	5.0 ± 1.0	3.7 ± 0.4 **	2.3 ± 0.3 **
Chemical intake	(mg/kg/day)	-	170 ± 51	387 ± 83	898 ± 203	1620 ± 172	2669 ± 261

Values indicate mean ± S.D.

Significant difference; *: $p \leqq 0.05$ **: $p \leqq 0.01$ by Dunnett's test.

a: One animal died during the 13-week administration period.

3). Urinary pH was decreased in male rats given 4,000 ppm and above, in the female rats given 10,000 ppm and 25,000 ppm, and in mice of both sexes given 10,000 ppm and 25,000 ppm (Data not shown).

Histopathology

The upper and lower respiratory tracts, liver, kidneys and brain were affected by oral administration of 1,4-dioxane in drinking water to rats (Table 4-1). The nuclear enlargement occurring in the nasal respiratory epithelium of male and female rats given 1,600 ppm and above was the most sensitive, and followed by the enlarged nuclei of epithelial cells in the olfactory epithelium and in the tracheal and bronchial epithelia. The nuclear enlargement was morphologically featured by the appearance of epithelial cells having round to oval nuclei which were at least 4 times as large in diameter as the normal nuclei of epithelial cells in the upper and lower respiratory tracts. The enlarged nuclei of the respiratory epithelial cells were distributed over the entire respiratory region at Levels 1 through 3 in the nasal cavity (Fig. 1). The enlarged nuclei in the olfactory epithelium occurring in the sustentacular cells were distributed over the entire olfactory region at Levels 2 and 3 (Fig. 2). The rat nuclear enlargement in the bronchial epithelium was localized only in the main bronchus.

Centrilobular swelling of hepatocytes was the most sensitive sign appearing in the male rats given 1,600 ppm

Table 2. Terminal body weight and relative organ weights of the rats and mice orally administered 1,4-dioxane in drinking water at 5 different concentrations or vehicle water for 13 weeks.

(A) Rats

Group (ppm)		Control	640	1600	4000	10000	25000
<Male>							
No. of animals examined		10	10	10	10	10	10
Body weight	(g)	331 ± 13	335 ± 9	337 ± 7	322 ± 15	309 ± 7 **	263 ± 22 **
Liver	(%)	2.464 ± 0.071	2.492 ± 0.073	2.520 ± 0.077	2.550 ± 0.072	2.571 ± 0.040 *	2.727 ± 0.118 **
Kidneys	(%)	0.587 ± 0.016	0.592 ± 0.021	0.600 ± 0.020	0.616 ± 0.021 **	0.626 ± 0.017 **	0.700 ± 0.040 **
Lungs	(%)	0.300 ± 0.019	0.303 ± 0.019	0.299 ± 0.013	0.313 ± 0.012	0.315 ± 0.010	0.346 ± 0.016 **
<Female>							
No. of animals examined		10	10	10	10	10	9 [a]
Body weight	(g)	194 ± 6	197 ± 7	188 ± 8	183 ± 7 **	172 ± 7 **	155 ± 7 **
Liver	(%)	2.332 ± 0.049	2.355 ± 0.084	2.569 ± 0.120 **	2.455 ± 0.074 *	2.599 ± 0.076 **	2.886 ± 0.110 **
Kidneys	(%)	0.602 ± 0.025	0.616 ± 0.026	0.664 ± 0.043 **	0.671 ± 0.039 **	0.771 ± 0.045 **	0.861 ± 0.030 **
Lungs	(%)	0.391 ± 0.021	0.381 ± 0.016	0.403 ± 0.025	0.408 ± 0.022	0.431 ± 0.018 **	0.451 ± 0.018 **

(B) Mice

Group (ppm)		Control	640	1600	4000	10000	25000
<Male>							
No. of animals examined		10	10	10	10	10	9 [a]
Body weight	(g)	30.9 ± 2.6	32.3 ± 3.0	31.3 ± 3.3	30.7 ± 4.0	29.4 ± 2.5	22.2 ± 2.0 **
Liver	(%)	3.642 ± 0.261	3.700 ± 0.212	3.794 ± 0.208	3.852 ± 0.231	3.763 ± 0.162	3.871 ± 0.366
Kidneys	(%)	1.407 ± 0.120	1.390 ± 0.113	1.438 ± 0.147	1.440 ± 0.134	1.504 ± 0.094	1.821 ± 0.063 **
Lungs	(%)	0.453 ± 0.042	0.469 ± 0.087	0.456 ± 0.048	0.478 ± 0.055	0.505 ± 0.050	0.767 ± 0.107 **
<Female>							
No. of animals examined		10	10	10	10	10	10
Body weight	(g)	20.0 ± 1.2	20.5 ± 1.1	20.3 ± 1.1	21.0 ± 1.6	20.8 ± 1.6	19.5 ± 1.2
Liver	(%)	4.587 ± 0.265	4.392 ± 0.186	4.593 ± 0.277	4.509 ± 0.190	4.386 ± 0.341	4.312 ± 0.105 *
Kidneys	(%)	1.456 ± 0.115	1.430 ± 0.107	1.473 ± 0.068	1.436 ± 0.099	1.549 ± 0.105	1.873 ± 0.421 **
Lungs	(%)	0.713 ± 0.067	0.698 ± 0.067	0.704 ± 0.059	0.702 ± 0.042	0.900 ± 0.070 **	1.049 ± 0.061 **

Values indicate mean ± S.D.

Significant difference; *: $p \leqq 0.05$ **: $p \leqq 0.01$ by Dunnett's test.

%; relative organ weight (absolute organ weight / terminal body weight × 100).

a: One animal died during the 13-week administration period.

and above (Fig. 3). Single cell necrosis, which accompanied inflammatory cell infiltration, significantly increased in male rats given 4,000 ppm and 25,000 ppm and in female rats given 25,000 ppm. A degenerative change such as vacuolic change in centrilobular hepatocytes occurred at high dose levels. Notably, altered hepatocellular foci stained positively with the anti-GST-P antibody were found in all the five 25,000 ppm-dosed rats of both sexes (Fig. 4), whereas there were no GST-P-positive foci in the livers of the five male or five female controls each.

Table 3. Hematology and blood biochemistry of the rats and mice orally administered 1,4-dioxane in drinking water at 5 different concentrations or vehicle water for 13 weeks.

(A) Rats

Group (ppm)	Control	640	1600	4000	10000	25000	
<Male>							
No. of animals examined	10	10	10	10	10	10	
Red blood cell (10^6/μl)	9.61 ± 0.24	9.66 ± 0.25	9.70 ± 0.21	9.59 ± 0.29	9.72 ± 0.24	9.96 ± 0.28	*
Hemoglobin (g/dl)	16.2 ± 0.2	16.1 ± 0.3	16.1 ± 0.3	16.0 ± 0.3	16.4 ± 0.4	16.6 ± 0.3	*
Hematocrit (%)	43.4 ± 1.3	43.4 ± 1.2	43.3 ± 0.9	43.0 ± 1.5	43.9 ± 1.2	45.0 ± 1.7	*
AST (IU/l)	75 ± 16	79 ± 15	80 ± 9	78 ± 11	83 ± 6	104 ± 15	**
ALT (IU/l)	26 ± 5	27 ± 4	29 ± 3	28 ± 3	29 ± 2	43 ± 9	**
Glucose (mg/dl)	181 ± 23	179 ± 20	177 ± 10	177 ± 18	164 ± 12	138 ± 14	**
<Female>							
No. of animals examined	10	10	9 [a]	10	10	9 [b]	
Red blood cell (10^6/μl)	8.82 ± 0.32	8.86 ± 0.23	8.77 ± 0.26	8.82 ± 0.17	8.76 ± 0.27	8.94 ± 0.27	
Hemoglobin (g/dl)	16.3 ± 0.5	16.3 ± 0.4	16.2 ± 0.4	16.2 ± 0.3	16.0 ± 0.5	16.2 ± 0.5	
Hematocrit (%)	43.1 ± 1.6	43.0 ± 1.1	42.8 ± 1.2	43.0 ± 0.7	42.2 ± 1.5	42.6 ± 1.4	
AST (IU/l)	67 ± 9	65 ± 10	67 ± 7	69 ± 7	68 ± 3	81 ± 6	**
ALT (IU/l)	21 ± 4	20 ± 3	20 ± 3	21 ± 3	21 ± 1	24 ± 2	
Glucose (mg/dl)	150 ± 14	155 ± 11	149 ± 8	144 ± 11	138 ± 11	124 ± 15	**

(B) Mice

Group (ppm)	Control	640	1600	4000	10000	25000	
<Male>							
No. of animals examined	9 [a]	10	10	9 [a]	10	9 [b]	
Red blood cell (10^6/μl)	10.67 ± 0.41	10.77 ± 0.34	10.39 ± 0.45	10.70 ± 0.42	10.91 ± 0.44	11.31 ± 0.51	*
Hemoglobin (g/dl)	14.9 ± 0.5	15.0 ± 0.3	14.6 ± 0.4	15.1 ± 0.4	15.4 ± 0.6	16.3 ± 0.6	**
Hematocrit (%)	43.3 ± 1.8	44.0 ± 1.5	42.6 ± 1.7	44.1 ± 1.7	45.2 ± 1.9	47.6 ± 1.5	**
AST (IU/l)	48 ± 10	49 ± 11	44 ± 8	43 ± 10	44 ± 6	70 ± 12	**
ALT (IU/l)	11 ± 2	13 ± 3	10 ± 2	12 ± 2	13 ± 2	25 ± 9	**
Glucose (mg/dl)	209 ± 37	207 ± 35	193 ± 31	190 ± 31	188 ± 25	132 ± 11	**
<Female>							
No. of animals examined	9 [a]	10	10	9 [a]	10	9 [a]	
Red blood cell (10^6/μl)	10.20 ± 0.37	10.21 ± 0.38	10.47 ± 0.42	10.52 ± 0.34	10.43 ± 0.66	10.45 ± 0.33	
Hemoglobin (g/dl)	14.6 ± 0.5	14.7 ± 0.5	15.0 ± 0.6	15.1 ± 0.6	15.3 ± 0.9	15.4 ± 0.4	
Hematocrit (%)	42.3 ± 1.6	42.5 ± 1.7	43.6 ± 1.9	43.6 ± 1.9	44.1 ± 2.8	44.7 ± 1.4	
AST (IU/l)	88 ± 19	87 ± 30	89 ± 18	87 ± 29	93 ± 14	139 ± 35	**
ALT (IU/l)	17 ± 4	17 ± 5	20 ± 5	22 ± 6	30 ± 6	** 50 ± 8	**
Glucose (mg/dl)	151 ± 14	151 ± 27	139 ± 17	136 ± 15	130 ± 15	* 123 ± 10	**

Values indicate mean ± S.D.

Significant difference; *: $p \leqq 0.05$ **: $p \leqq 0.01$ by Dunnett's test.

AST: Aspartate aminotransferase. ALT: Alanine aminotransferase.

a: The measurement could not be performed for 1 animal because of shortage of blood volume.

b: One animal died during the 13-week administration period.

Table 4-1. Number of rats bearing the lesions in the respiratory tract, liver, kidneys and brain. The animals were orally administered 1,4-dioxane in drinking water at 5 different concentrations or vehicle water for 13 weeks.

	Male						Female					
Group (ppm)	Control	640	1600	4000	10000	25000	Control	640	1600	4000	10000	25000
No. of animals examined	10	10	10	10	10	10	10	10	10	10	10	9 [a]
< Nasal cavity >												
Nuclear enlargement: respiratory epithelium	0	0	9 **	10 **	9 **	10 **	0	0	5 *	10 **	10 **	8 **
			(1.0)	(2.0)	(2.0)	(2.0)			(1.0)	(1.0)	(1.0)	(1.0)
Nuclear enlargement: olfactory epithelium	0	0	0	10 **	9 **	10 **	0	0	0	9 **	10 **	8 **
				(1.0)	(1.0)	(2.0)				(1.0)	(1.0)	(1.0)
< Trachea >												
Nuclear enlargement: epithelium	0	0	0	10 **	10 **	10 **	0	0	0	9 **	10 **	9 **
				(1.0)	(1.0)	(2.8)				(1.0)	(1.0)	(2.1)
< Bronchus >												
Nuclear enlargement: bronchial epithelium	0	0	0	0	1	2	0	0	0	1	1	6 *
					(1.0)	(1.0)				(1.0)	(1.0)	(1.0)
< Liver >												
Necrosis: single cells	0	0	0	5 *	2	10 **	2	0	1	5	5	8 **
				(1.0)	(1.0)	(1.1)	(1.0)		(1.0)	(1.0)	(1.2)	(1.5)
Swelling: centrilobular	0	0	9 **	10 **	10 **	10 **	0	0	1	0	9 **	9 **
			(1.0)	(1.1)	(2.0)	(2.9)			(1.0)		(1.0)	(1.7)
Vacuolic change: centrilobular	0	0	1	0	10 **	10 **	0	0	0	0	0	9 **
			(1.0)		(1.5)	(3.0)						(2.2)
< Kidney >												
Hydropic change: proximal tubule	0	0	0	0	0	7 **	0	0	0	0	0	5 *
						(1.0)						(1.6)
Nuclear enlargement: proximal tubule	0	0	0	1	5 *	9 **	0	0	0	0	8 **	9 **
				(1.0)	(1.0)	(1.0)					(1.0)	(1.0)
< Brain >												
Vacuolic change	0	0	0	0	0	10 **	0	0	0	0	0	9 **
						(1.2)						(1.0)

Note: Values indicate number of animals bearing lesion.

The values in parentheses indicate the average of severity grade index of the lesion. The average of severity grade was calculated with the following equation. Σ(grade × number of animals with grade) / number of affected animals. Grade: 1 = slight, 2 = moderate, 3 = severe.

Significant difference; *: $p \leqq 0.05$ **: $p \leqq 0.01$ by chi-square test.

a: One dead animal was excluded for the analysis.

Table 4-2. Number of mice bearing the lesions in the respiratory tract and liver. The animals were orally administered 1,4-dioxane in drinking water at 5 different concentrations or vehicle water for 13 weeks.

	Male						Female					
Group (ppm)	Control	640	1600	4000	10000	25000	Control	640	1600	4000	10000	25000
No. of animals examined	10	10	10	10	10	9[a]	10	10	10	10	10	10
< Nasal cavity >												
Nuclear enlargement: respiratory epithelium	0	0	0	2	5	0	0	0	0	3	3	7 **
				(1.0)	(1.0)					(1.0)	(1.0)	(1.1)
Nuclear enlargement: olfactory epithelium	0	0	0	9 **	10 **	9 **	0	0	0	6 *	10 **	10 **
				(1.0)	(1.1)	(1.0)				(1.0)	(1.5)	(2.0)
Vacuolic change: olfactory nerve	0	0	0	0	0	9 **	0	0	0	0	2	8 **
						(1.1)					(1.0)	(1.3)
< Trachea >												
Nuclear enlargement: epithelium	0	0	0	7 **	9 **	9 **	0	0	2	9 **	10 **	10 **
				(1.0)	(1.0)	(1.1)			(1.0)	(1.0)	(1.0)	(1.3)
< Bronchus >												
Nuclear enlargement: bronchial epithelium	0	0	0	9 **	9 **	9 **	0	0	10 **	10 **	10 **	10 **
				(1.0)	(1.0)	(1.4)			(1.0)	(1.1)	(2.1)	(3.0)
Degeneration: bronchial epithelium	0	0	0	0	0	8 **	0	0	0	0	7 **	10 **
						(1.1)					(1.0)	(1.1)
< Liver >												
Necrosis: single cells	0	0	0	5 *	10 **	9 **	0	0	0	7 **	10 **	9 **
				(1.0)	(1.0)	(1.0)				(1.0)	(1.0)	(1.0)
Swelling: centrilobular	0	0	0	10 **	10 **	9 **	0	1	1	10 **	10 **	9 **
				(1.1)	(1.0)	(2.0)		(1.0)	(1.0)	(1.0)	(1.0)	(2.0)

Note: Values indicate number of animals bearing lesion.

The values in parentheses indicate the average of severity grade index of the lesion. The average of severity grade was calculated with the following equation. Σ(grade × number of animals with grade) / number of affected animals. Grade: 1 = slight, 2 = moderate, 3 = severe.

Significant difference; *: $p \leqq 0.05$ **: $p \leqq 0.01$ by chi-square test.

a: One dead animal was excluded for the analysis.

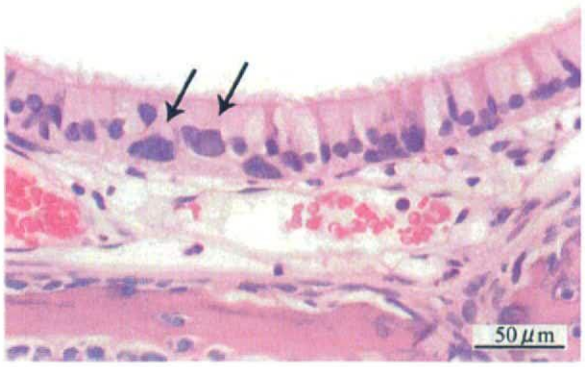

Fig. 1. Nuclear enlargement (arrows) in the respiratory epithelium at Level 3 of a male rat given 25,000 ppm 1,4-dioxane in drinking water for 13 weeks. H and E stain.

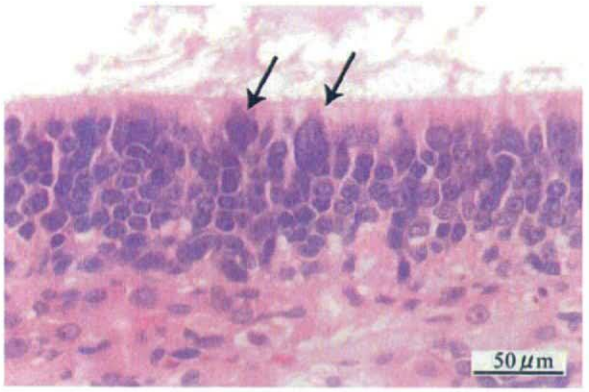

Fig. 2. Nuclear enlargement of the sustentacular cells (arrows) in the olfactory epithelium at Level 3 of a male rat given 25,000 ppm 1,4-dioxane in drinking water for 13 weeks. H and E stain.

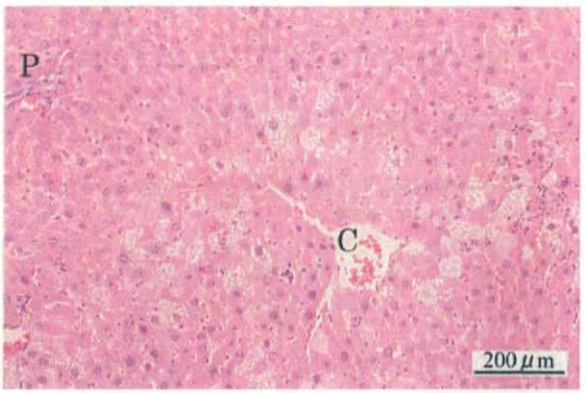

Fig. 3. Centrilobular swelling and vacuolic change of hepatocytes of a male rat given 25,000 ppm 1,4-dioxane in drinking water for 13 weeks. Portal triad (P). Central vein (C). H and E stain.

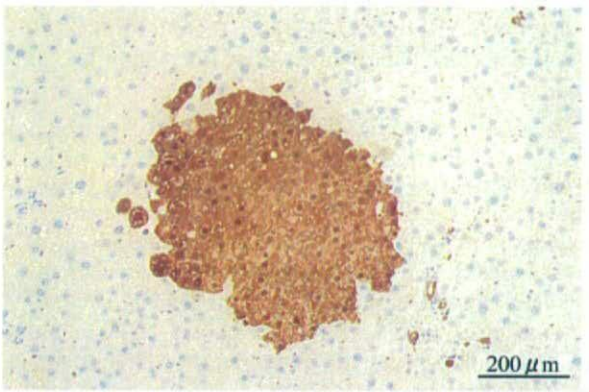

Fig. 4. A GST-P-positive focus in the liver of a male rat given 25,000 ppm 1,4-dioxane in drinking water for 13 weeks. Immunochemical staining with GST-P.

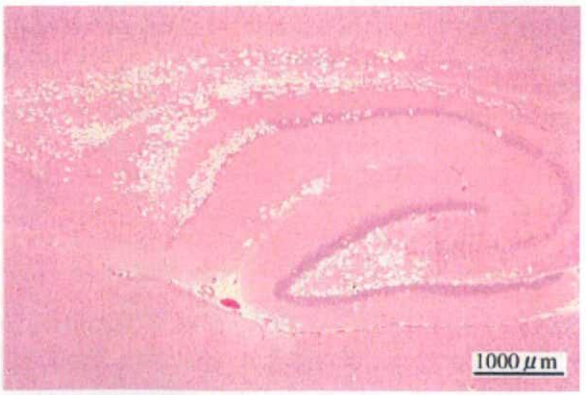

Fig. 5. Vacuolic change in the cerebrum of a male rat given 25,000 ppm 1,4-dioxane in drinking water for 13 weeks. The lesion is located in the corpus callosum, hippocampus and dentate gyrus. H and E stain.

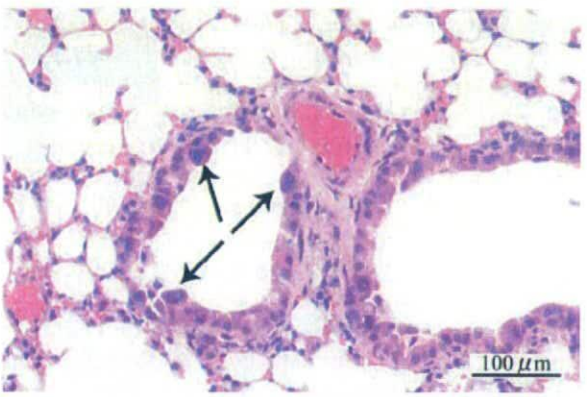

Fig. 6. Nuclear enlargement (arrows) in the bronchial epithelium of a male mouse given 25,000 ppm 1,4-dioxane in drinking water for 13 weeks. H and E stain.

However, the hematoxylin-eosin stained altered hepatocellular foci could not be clearly detected in any 1,4-dioxane-dosed rat of either sex.

Nuclear enlargement of epithelial cells in the renal proximal tubules characterized by the appearance of epithelial cells having nuclei which were up to about 2 times as large as the normal nucleus in diameter occurred in the male and female rats given 10,000 ppm and 25,000 ppm, while hydropic change in the proximal tubules was observed only in the 25,000 ppm-dosed males and females.

Vacuolic change in the cerebrum was noted in the 25,000 ppm-dosed males and females. The cerebral vacuoles varied in size, up to about 100 μm, and were located in the corpus callosum, hippocampus and dentate gyrus (Fig. 5).

In the 1,4-dioxane-dosed mice, positive histopathological findings were observed in the upper and lower respiratory tracts and liver (Table 4-2). Like the 1,4-dioxane-dosed rats, the mouse nuclear enlargement was also a characteristic sign in the upper and lower respiratory tracts. The morphological features of the nuclear enlargement in mice were similar to those in rats, but the nuclear size was about 2 times as large in diameter as that in normal nuclei of epithelial cells. The nuclear enlargement appearing in the bronchial epithelium of female mice given 1,600 ppm and above was the most sensitive, while the significantly increased incidence of the nuclear enlargement was observed in both the olfactory epithelium and trachea of male and female mice given 4,000 ppm and above, and in the bronchial epithelium of male mice given 4,000 ppm and above, and in the respiratory epithelium of female mice given 25,000 ppm. Distribution of the mouse nuclear enlargement was essentially similar to that of the rat nuclear enlargement, except for the bronchial epithelium in which the enlarged nuclei were widely distributed over the lower respiratory tract from the main bronchus to terminal bronchioles (Fig. 6). In addition to the nuclear enlargement, vacuolic change in the olfactory nerve cells and degeneration in the bronchial epithelium were noted in the male and female mice given 25,000 ppm, while the latter degeneration also occurred in the females given 10,000 ppm. The vacuolic change in the olfactory nerve cells was mainly observed in the nerve bundles in the lamina propria of dorsal wall at Levels 2 and 3. The degeneration of the bronchial epithelium occurred throughout from the main bronchus to the terminal bronchiole. Both single cell necrosis and swelling of centrilobular hepatocytes were significantly increased in male and female mice given 4,000 ppm and above.

DISCUSSION

In the present study, oral administration of 1,4-dioxane in drinking water for 13 weeks was found to affect the upper and lower respiratory tracts and liver in rats and mice of both sexes. In addition, the kidneys and brain in rats of both sexes were affected. Nuclear enlargement in the nasal respiratory epithelium was the most sensitive, appearing in the rats of both sexes given 1,600 ppm and above. The rat nuclear enlargement in the olfactory, tracheal and bronchial epithelia appeared to lesser extent than that in the respiratory epithelium. The nuclear enlargement was morphologically featured by the appearance of the epithelial cells having round to oval nuclei which were at least 4 times as large in diameter as the normal nuclei of epithelial cells in the upper and lower respiratory tracts. The enlarged nuclei of the respiratory and olfactory epithelial cells were distributed over the entire respiratory and olfactory region, respectively. The nuclear enlargement was also observed in the renal proximal tubules of the male and female rats given 10,000 ppm and 25,000 ppm. It was noteworthy that the rat nuclear enlargement of epithelial cells in the respiratory tract was essentially similar in sensitivity and distribution to the mouse nuclear enlargement except for the bronchial epithelium. The enlarged nuclei in the mouse bronchial epithelium were widely distributed over the entire region tract from the main bronchus to terminal bronchioles, while the rat nuclear enlargement was localized in the main bronchus. The nuclear enlargement was reported to occur as an early histopathological change induced by chemical carcinogens in the upper respiratory tract of rats exposed to sulphur dioxide and intraperitoneally injected with several *N*-nitrosamines which are known to induce nasal tumors in rats (Fowlie *et al.*, 1990). Grant and Grasso (1978) reported that there is a good correlation between *in vivo* carcinogenicity and the extent of nuclear enlargement in HeLa cells *in vitro*. Therefore, the nuclear enlargement found in the present study might be causally related to the possible development of carcinogenicity by 1,4-dioxane in the upper respiratory tract of rats and mice.

Since in the present study 1,4-dioxane was orally administered to rats and mice through water drinking, the nuclear enlargement might be primarily caused by the 1,4-dioxane which was conveyed to the epithelial tissues through the blood stream after the gastro-intestinal absorption and by exhalation passing back through the nasal cavity from the alveolar space during the gas exchange process. This working hypothesis can be supported by the wide distribution of the enlarged nuclei over the entire

upper respiratory tract including the nasal cavity and trachea, in the absence of localization of the enlarged nuclei in the anterior and dorsal part of the nasal epithelia that the inhaled air first contacts. However, a species difference in sensitivity and distribution of the nuclear enlargement in the bronchial epithelium between rats and mice could not be accounted for in the term of the 1,4-dioxane in the blood stream. In this context, the formation of toxic metabolites of the blood-circulated 1,4-dioxane by the enriched xenobiotic enzymes in the nasal cavity might also be causally related to the wide distribution of nuclear enlargement in the upper respiratory tract, since the nasal epithelium is enriched with xenobiotic enzymes in rodents (Dahl and Hadley, 1991), and since 1,4-dioxane is metabolized to beta-hydroxyethoxy acetic acid and 1,4-dioxane-2-one by the liver mixed-function oxidase (Woo *et al.*, 1978; Young *et al.*, 1978).

Centrilobular swelling of hepatocytes was the most sensitive among the three hepatic lesions of rats, and was as sensitive as the nuclear enlargement in the respiratory epithelium, both of which occurred in the male rats given 1,600 ppm. Single cell necrosis of hepatocytes appeared in both 25,000 ppm-dosed male and female rats and in the 4,000 ppm-dosed male rats. Vacuolic change of hepatocytes and increased cytolytic release of liver-associated transaminases into plasma occurred at high dose levels. On the other hand, the mouse hepatic lesions were characterized by both single cell necrosis and centrilobular swelling of hepatocytes, occurring at 4,000 ppm and above. Increased cytolytic release of liver-associated transaminases into plasma as a biochemical sign of degenerative or necrotic hepatocytes was observed at higher dose levels than the hepatocellular necrosis in the 1,4-dioxane-dosed mice. It is of interest to note that the oral administration of 1,4-dioxane in drinking water for 13 weeks did induce the GST-P-positive altered hepatocellular foci in the 1,4-dioxane-dosed rats of both sexes. The 1,4-dioxane-induced, GST-P-positive foci are considered to be derived clonally from the single "1,4-dioxane-initiated" hepatocytes, and are consistent with morphological features of the GST-P-positive hepatocellular foci initiated by diethylnitrosamine, a potent initiator (Moore *et al.*, 1987; Tatematsu *et al.*, 1985; Ito *et al.*, 1997). Lundberg *et al.* (1987) reported that repeated oral administration of 1,4-dioxane by gavage to rats increased the number and total volume of gamma-glutamyltranspeptidase-positive hepatocellular foci in the rats which had been initiated with diethylnitrosamine after partial hepatectomy. Taken together, it is suggested that 1,4-dioxane has either initiating or promoting activity. Since the GST-P-positive foci have been recognized as the preneoplastic lesion that allows the prediction of hepatocarcinogenicity with high probability (Ito *et al.*, 1988, 1997, 2000), the occurrence of the GST-P-positive altered hepatocellular foci suggests that prolonged oral administration of 1,4-dioxane in drinking water for up to 2 years causes hepatocellular tumors, as indicated by the previous preliminary report (Yamazaki *et al.*, 1994).

It was found in the present study that the most sensitive sign of subchronic toxicity of 1,4-dioxane was the nuclear enlargement in the nasal respiratory epithelium, which occurred in the male and female rats given 1,600 ppm. In addition, the centrilobular swelling of hepatocytes appeared in the male rats given 1,600 ppm. As the most sensitive sign in mice, the 1,600 ppm-dosed female mice were found to exhibit a significantly increased incidence of nuclear enlargement in the bronchial epithelium. In the male mice, however, the significantly increased incidences of nuclear enlargement in the olfactory, tracheal and bronchial epithelia, and of single cell necrosis and swelling of centrilobular hepatocytes in the liver were noted at 4,000 ppm. Therefore, a NOAEL can be determined at 640 ppm in the male and female rats and female mice, and at 1,600 ppm in the male mice. 1,4-Dioxane intake in the 640 ppm groups of male and female rats and female mice, and in the 1,600 ppm group of male mice was estimated as 52, 83, 170 and 231 mg/kg/day, respectively. It was concluded that the NOAEL value was 52 mg/kg/day in rats and 170 mg/kg/day in mice.

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Granulocyte/macrophage colony-stimulating factor autoantibodies and myeloid cell immune functions in healthy subjects

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Granulocyte/macrophage–colony-stimulating factor autoantibodies and myeloid cell immune functions in healthy subjects

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High levels of granulocyte/macrophage–colony-stimulating factor (GM-CSF) autoantibodies are thought to cause pulmonary alveolar proteinosis (PAP), a rare syndrome characterized by myeloid dysfunction resulting in pulmonary surfactant accumulation and respiratory failure. Paradoxically, GM-CSF autoantibodies have been reported to occur rarely in healthy people and routinely in pharmaceutical intravenous immunoglobulin (IVIG) purified from serum pooled from healthy subjects. These findings suggest that either GM-CSF autoantibodies are normally present in healthy people at low levels that are difficult to detect or that serum pooled for IVIG purification may include asymptomatic persons with high levels of GM-CSF autoantibodies. Using several experimental approaches, GM-CSF autoantibodies were detected in all healthy subjects evaluated (n = 72) at low levels sufficient to rheostatically regulate multiple myeloid functions. Serum GM-CSF was more abundant than previously reported, but more than 99% was bound and neutralized by GM-CSF autoantibody. The critical threshold of GM-CSF autoantibodies associated with the development of PAP was determined. Results demonstrate that free serum GM-CSF is tightly maintained at low levels, identify a novel potential mechanism of innate immune regulation, help define the therapeutic window for potential clinical use of GM-CSF autoantibodies to treat inflammatory and autoimmune diseases, and have implications for the pathogenesis of PAP. (Blood. 2009;113:2547-2556)

Introduction

Granulocyte/macrophage–colony-stimulating factor (GM-CSF) is a pleiotropic cytokine regulator of myeloid and other immune and nonimmune cells that is required for terminal differentiation of alveolar macrophages in the lungs and regulates the basal functional capacity of circulating neutrophils in mice and humans.[1-7] The paracrine,[3,8] autocrine,[9] and endocrine[10] effects of GM-CSF are mediated via heterologous cell-surface receptors[11] reported to stimulate myeloid cell survival at low GM-CSF concentrations, and survival, proliferation, differentiation, and antimicrobial functions at high concentrations.[12] Normally, GM-CSF is present at very low or undetectable levels in the serum and tissues in both mice and humans.[5,13] Nonetheless, these low levels are critical because GM-CSF–deficient mice have impaired myeloid cell functions, increased mortality from microbial infections, and a lung phenotype characterized by progressive surfactant accumulation as a result of impaired alveolar macrophage surfactant catabolism.[3,5,14-17]

Autoimmune pulmonary alveolar proteinosis (PAP) is a human disease characterized by high levels of GM-CSF autoantibodies and respiratory insufficiency as a result of pulmonary surfactant accumulation[4,18,19] with features similar in nearly every respect to those seen in GM-CSF knockout mice.[3] Disease pathogenesis is thought to be mediated by GM-CSF autoantibodies, which eliminate GM-CSF bioactivity[20] and impair GM-CSF–dependent myeloid cell functions.[5]

Sustained elevation of GM-CSF also seems to be detrimental because transgenic mice nonspecifically overexpressing GM-CSF develop a fatal syndrome of myeloproliferation and inflammation-related tissue destruction.[21] Furthermore, increased local expression of GM-CSF occurs in rheumatoid arthritis in humans, and neutralization of GM-CSF ameliorates disease development in animal models of rheumatoid arthritis[22] and multiple sclerosis,[23] indicating that GM-CSF may be involved in the pathogenesis of inflammatory and autoimmune diseases.[24] These findings strongly suggest that GM-CSF is tightly maintained at very low but critical levels in both humans and mice.

GM-CSF autoantibodies are consistently detected and comprise the major anti-cytokine activity in pharmaceutical intravenous immunoglobulin (IVIG) prepared from pooled serum of healthy subjects.[25] In contrast, GM-CSF autoantibodies have been rarely detected in the serum of healthy persons[25] and, when present, levels were far lower than in patients with PAP.[26] These seemingly paradoxical findings suggest that the pooled serum used to prepare pharmaceutical IVIG may include serum from perons who seem

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