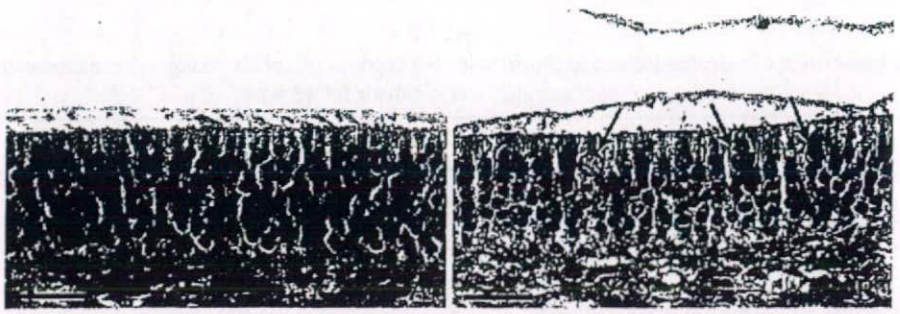

FIG. 2. (A) The normal olfactory epithelium in a male rat exposed to clean air for 13 wk. (B) Nuclear enlargement (arrows) of sustentacular cells and vacuolic change (arrowhead) of the olfactory sensory cells in the olfactory epithelium at Level 3 of nasal cavity in a male rat exposed to 3200 ppm 1,4-dioxane for 13 wk. H&E stain. Bars indicate 50 μm.

four 1600-ppm-exposed females out of each ten 1,4-dioxane-exposed rats of either sex, whereas the GST-P-positive foci could not be found in any of the 800- and 1600-ppm-exposed males and 800-ppm-exposed females and control groups of both sexes. The GST-P-positive hepatocytes exhibited morphologically focal and clonal proliferation.

The incidence of hydropic change in the renal proximal tubules was significantly increased in the 3200-ppm-exposed females.

Blood Levels of 1,4-Dioxane

Plasma levels of 1,4-dioxane were detected in the concentrations of exposure to 400 ppm and above, and increased linearly with the increase in the exposure concentration. The mean plasma levels of 1,4-dioxane in the 400-, 800-, 1600-, and 3200-ppm-exposed rats were 48, 152, 319, and 730 μg/ml for males, and 80, 209, 468, and 1054 μg/ml for females, respectively. Plasma levels of 1,4-dioxane were higher in females than in males. Figure 4 shows a highly linear relationship for each sex between the plasma levels of 1,4-dioxane and concentrations of inhalation exposure to 1,4-dioxane at 400 ppm and above, as indicated by the adjusted coefficient R^2 values greater than .9.

DISCUSSION

In this study, repeated inhalation exposure to 1,4-dioxane vapor for 13 wk was found to affect the upper and lower respiratory tracts and liver in rats of both sexes and kidneys in female rats. Nuclear enlargement of epithelial cells in the nasal respiratory epithelium was the most sensitive lesion occurring in the 100-ppm-exposed males and females. The incidence and severity of enlarged nuclei of epithelial cells decreased along the passage of 1,4-dioxane-containing inspiratory flow through the upper and lower respiratory tracts, suggesting that the nuclear enlargement tended to decrease with the presumably gradual decrease in the amount of 1,4-dioxane absorbed in the mucous layer of the respiratory, olfactory, tracheal, and bronchial epithelia. Notably, oral administration of 1,4-dioxane in drinking water for 13 wk was also found to induce nuclear enlargement in the nasal epithelia

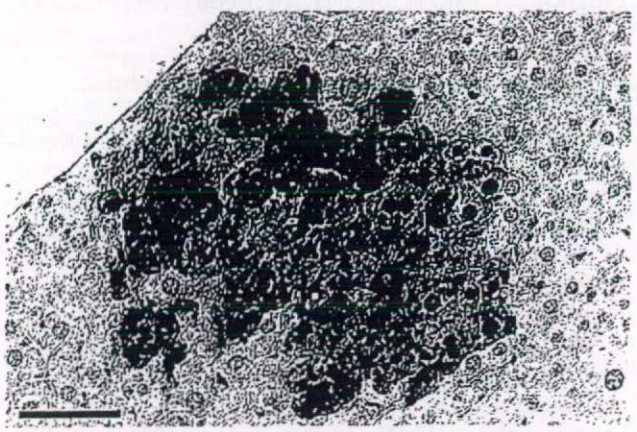

FIG. 3. A GST-P-positive focus in the liver of a male rat exposed to 3200 ppm 1,4-dioxane for 13 wk. Immunochemical staining with GST-P antibody. Bar indicates 100 μm.

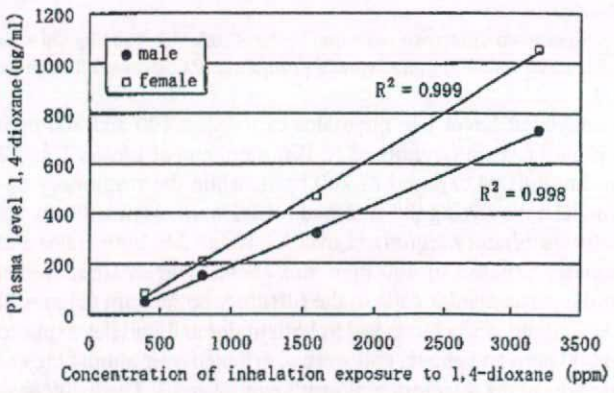

FIG. 4. Plasma levels of 1,4-dioxane as a function of the concentrations of exposure to 1,4-dioxane vapor. Filled circles and open squares represent the mean values of male and female rats, respectively.

of rats and mice of both sexes (Kano et al., 2008). However, there is a clear difference in the location of the 1,4-dioxane-induced enlarged nuclei of the nasal epithelial cells between the inhalation exposure and the oral administration. The respiratory epithelial area having the enlarged nuclei was expanded from the anterior portion (Level 1) to the entire region (Levels 1, 2, and 3) with an increase in the concentrations of inhalation exposure to 1,4-dioxane from 100 ppm to 3200 ppm. On the other hand, the oral administration of 1,4-dioxane-formulated drinking water uniformly produced the nuclear enlargement over the entire region (Levels 1, 2, and 3) of the respiratory epithelium without any anterior–posterior gradient along the nasal passage. This difference in the route of exposure can be accounted for in terms of a first-pass effect such that the inhaled 1,4-dioxane comes into first contact with the anterior portion of the respiratory epithelium, while the orally administered 1,4-dioxane is conveyed to the respiratory epithelial cells through the nasal blood flow after its first entrance in the gastrointestinal system including the liver. The nuclear enlargement was reported to occur as an early histopathological change in the upper respiratory tract of the rat simultaneously exposed to sulfur dioxide and intraperitoneally injected with several *N*-nitrosamines that are known to induce nasal tumors in rats (Fowlie et al., 1990). Grant and Grasso (1978) showed a good correlation between in vivo carcinogenicity and the extent of nuclear enlargement in HeLa cells in vitro. It can be inferred, therefore, that the nuclear enlargement of nasal epithelial cells found in the present study represents an early detectable and potentially preneoplastic lesion participating in the possible development of nasal tumors that would be induced by 2-yr inhalation exposure of rats to 1,4-dioxane vapor.

A significant increase in the degenerative change was evident in the olfactory epithelium of the males exposed to 400 ppm and above and of the females exposed to 800 ppm and above. The degenerative change was characterized by a vacuolic change and a decreased number of olfactory sensory cells, suggestive of possible impairment of olfactory sensation. Therefore, the olfactory epithelial degeneration is thought to represent a biologically significant adverse effect allowing extrapolation of animal toxicity data to humans, since the degenerative lesion of nasal mucosa might cause attenuation or loss of the olfactory acuity in humans (Tvedt et al., 1991; Hirsch & Zavala, 1999).

Another quantitative difference in the 1,4-dioxane-induced nasal and hepatic lesions between the present inhalation study and the oral administration study (Kano et al., 2008) can be recognized in light of the first-pass effect on the target organs. In the oral administration study, the nasal cavity lesions were induced at the drinking-water concentration of 1600 ppm, which corresponded to the estimated daily doses of 126 and 185 mg/kg in male and female rats, respectively. In the present inhalation study, however, the nasal cavity lesions were observed at the lowest inhalation exposure concentration of 100 ppmb (v/v), which corresponded to the estimated 1,4-dioxane uptake of 73 mg/kg/day, assuming that the minute volume is 561 ml/min/kg body weight for rats (Mauderly et al., 1979) and that the uptake ratio of 1,4-dioxane by the upper and lower respiratory tracts is 100%. Evidently, the 1,4-dioxane-induced nasal cavity lesions appeared at lower uptake levels by the inhalation exposure than by the oral administration. On the other hand, the oral administration of 1,4-dioxane induced centrilobular swelling of hepatocytes at the estimated daily uptake of 126 mg/kg in male rats and 756 mg/kg in female rats (Kano et al., 2008). The inhalation exposure to 1,4-dioxane induced centrilobular swelling of hepatocytes only at the highest exposure concentration of 3200 ppm (v/v), which corresponded to the estimated 1,4-dioxane uptake of 2336 mg/kg/day. Thus, the 1,4-dioxane-induced hepatic lesions appeared at lower uptake levels by oral administration than by inhalation exposure. These differences between oral administration and inhalation exposure can also be accounted for in the same terms of the first-pass effect on the gastrointestinal tract, including the liver, causing higher hepatic levels of 1,4-dioxane by oral administration than by inhalation exposure.

The hepatic lesions that occurred at higher concentrations of inhalation exposure to 1,4-dioxane than the nasal lesions did were characterized by both single-cell necrosis and hepatocellular swelling in the male rats and hepatocellular swelling in the females, in addition to GST-P-positive liver foci. The GST-P-positive hepatocytes appeared to be focally and clonally proliferating. It was reported, however, that a broad range of in vitro and in vivo genotoxicity assays for 1,4-dioxane produced negative results (IARC, 1999; Morita & Hayashi, 1998), and that no tumor was induced by 2-yr inhalation exposure of rats to 1,4-dioxane vapor at a level of 111 ppm (Torkelson et al., 1974). GST-P is known as a good specific marker enzyme for detecting an early histogenetic stage participating in the development of rat hepatocellular tumors by chemical carcinogens (Sato et al., 1984; Tatematsu et al., 1985; Ito et al., 1988). Therefore, a portion but not all of the GST-P-positive hepatocellular foci occurring after 13-wk inhalation exposure to 1,4-dioxane is considered to undergo further focal and clonal proliferation and progress finally to hepatocellular tumors that would be induced after 2-yr inhalation exposure to 1,4-dioxane at appropriately selected exposure concentrations in due consideration of the criterion of maximum tolerated dose (MTD) (Sontag et al., 1976; Bannasch et al., 1986). Indeed, the MTD for the 2-yr bioassay study for rat carcinogenicity of 1,4-dioxane is estimated to be much higher than the exposure concentration of 111 ppm used by Torkelson et al. (1974).

Inhalation exposure to 1,4-dioxane was found to severely affect the kidneys at high exposure concentrations, because the cause of deaths in the 6400-ppm-exposed rats of both sexes was attributed primarily to the renal failure resulting from marked necrosis in the renal tubules. The renal lesions of surviving animals were characterized by hydropic change in the proximal tubules occurring only in the 3200-ppm-exposed females but not in the males and by significantly increased relative kidney weight occurring in the 3200-ppm-exposed males and in the

females exposed to 800 ppm and above. Thus, higher susceptibility of females to the renal toxicity of 1,4-dioxane than that of males is suggested. This gender difference might be causally related to the following two factors. First, it was found in the present study that the mean plasma levels of 1,4-dioxane were higher in female rats than in males. Second, it is known that the level of CYP2E1 in the rat is greater in females than in males (Parkinson, 2001). Since Nannelli et al. (2005) demonstrated the induction of renal CYP2E1 enzymes in the rat receiving acute and chronic oral administration of 1,4-dioxane, higher susceptibility of females to the renal toxicity might be related to either the increased kidney levels of 1,4-dioxane expected from the increased plasma level of 1,4-dioxane in females over males or the enhanced production of its toxic metabolites by the renal CYP2E1 induction in females. However, the exact role of 1,4-dioxane or its metabolites involved in the mechanism of renal toxicity still remains unsolved (Woo et al., 1978; Hecht & Young, 1981; Nannelli et al., 2005).

A highly linear increase in plasma levels of 1,4-dioxane with an increase in the concentrations of inhalation exposure to 1,4-dioxane at up to 3200 ppm can be taken to indicate that the metabolic capacity is not saturated up to plasma levels of 1,4-dioxane at 730 μg/ml for male rats and 1,054 μg/ml for female rats. This result appears to be in sharp contrast to the Young et al. finding (1978) that single oral doses of 1,4-dioxane resulting in plasma levels above 100 μg/ml are removed from the body more slowly due to the saturation of metabolism. It was reported that 1,4-dioxane is metabolized by cytochrome P-450 (P450) enzymes and excreted primarily into urine as 2-hydroxyethoxyacetic acid or 1,4-dioxane-2-one (Braun & Young, 1977; Young et al., 1978; Woo et al., 1978; Nannelli et al., 2005). The 1,4-dioxane metabolism was reported to be enhanced by the induction of hepatic P450 enzymes in the rat pretreated with phenobarbital (Woo et al., 1978) and in the rat receiving repeated oral administration of 1,4-dioxane (Young et al., 1978; Nannelli et al., 2005). Nannelli et al. (2005) also reported that P450 2E1 enzymes in the nasal cavity and kidney are induced by repeated oral administration of 1,4-dioxane in drinking water. Therefore, lack of the metabolic saturation of 1,4-dioxane found in the present study might be attributed to the enhanced metabolism by the possible induction of P450 enzymes including CYP2E1 by 13-wk repeated inhalation exposure to 1,4-dioxane at up to 3200 ppm.

In the present inhalation study, a LOAEL value can be determined at 100 ppm for the nasal endpoint, because of the significantly increased incidence of nuclear enlargement in the nasal respiratory epithelium of both male and female rats exposed to the lowest exposure concentration of 100 ppm 1,4-dioxane. This LOAEL value is thought to be lower than the reported NOAEL of 400 mg 1,4-dioxane/m^3 (111 ppm) (EU Risk Assessment Report, 2002), which was based on Torkelson et al.'s report (1974). In the previous study of 13-wk oral administration of 1,4-dioxane (Kano et al., 2008), the most sensitive sign was both the nuclear enlargement in the nasal respiratory epithelium and the centrilobular swelling of hepatocytes. A NOAEL value was determined at a drinking-water concentration of 640 mg 1,4-dioxane/kg water for those 2 endpoints, which corresponded to the estimated 1,4-dioxane uptake of 52 and 83 mg/kg/day in male and female rats, respectively. The LOAEL value of 100 ppm (v/v) obtained by the daily 6-h inhalation exposure of F344 rats to 1,4-dioxane vapor for 13 wk corresponded to the estimated 1,4-dioxane uptake of 73 mg/kg body weight/day. Therefore, taking into consideration the difference in the definition between LOAEL and NOAEL (IPCS, 1994), the presumed NOAEL value expected from the LOAEL of 100 ppm obtained by the 13-wk inhalation exposure to 1,4-dioxane vapor is lower than the NOAEL obtained by the 13-wk oral administration. The present finding that the presumed NOAEL for the nasal response to the inhaled 1,4-dioxane is more sensitive than that for the same endpoint to the ingested 1,4-dioxane can also be attributable to the first-pass effect discussed earlier for the nasal cavity lesion, and therefore may implicate the importance of the nasal response as an endpoint that can be used for risk assessment of humans exposed to hazardous chemicals by inhalation.

Recommendation of the OEL value of 20 ppm for 1,4-dioxane by the ACGIH (2001) was based on both animal and human studies. For the animal basis of toxicity, the ACGIH used the NOAEL of 111 ppm, judging by the Torkelson et al. (1974) result that neither pathological changes nor tumors were induced on gross and microscopic examinations of organs and tissues in male and female Wistar rats exposed by inhalation to 1,4-dioxane vapor at 111 ppm for 7 h/day, 5 days/wk, for 104 wk, although the nasal cavity was not listed in the organs subjected to the microscopic examination in their report. The nasal-cavity lesions found in the 1,4-dioxane-inhaled rat are relevant to possible adverse health effects arising from workers' inhalation exposure to 1,4-dioxane, and the route of exposure and the daily and weekly exposure regimens employed in the present study are closely simulated for human exposure to 1,4-dioxane in workplaces. The presumed NOAEL value of 1,4-dioxane for the nasal endpoint is estimated to be lower than the LOAEL of 100 ppm found in the present inhalation study. Assuming an uncertainty factor of 10 for extrapolation of rodent data to humans (Barns, 1988), one-tenth of the presumed NOAEL value for 1,4-dioxane falls below the current OEL value of 20 ppm, suggesting the need to reconsider the OEL for 1,4-dioxane.

In conclusion, the present study demonstrated toxic effects in the upper and lower respiratory tract, liver, and kidneys in the rats exposed by inhalation to 1,4-dioxane vapor. Nuclear enlargement of the respiratory epithelial cells occurring at 100 ppm was the most sensitive lesion. The enlarged nuclei in the nasal epithelia might represent an early recognizable stage suggestive of the possible development of nasal tumors. The hepatic lesions that occurred in higher concentrations than did the nasal-cavity lesions were characterized by single-cell necrosis and centrilobular swelling of hepatocytes, in addition to the preneoplastic GST-P-positive liver foci. These results might predict that 2-yr

inhalation exposure to 1,4-dioxane induces nasal and hepatic tumors, suggestive of the urgent need for a 2-yr bioassay study of rodent carcinogenicity for 1,4-dioxane.

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Original Article

Enhanced proliferative response of hepatocytes to combined inhalation and oral exposures to *N,N*-dimethylformamide in male rats

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ABSTRACT — Male Wistar rats were exposed by inhalation to *N,N*-dimethylformamide (DMF) at 0 (control), 200 or 400 ppm (v/v) for 6 hr/day, 5 days/week and 4 weeks, and each inhalation group received DMF-formulated drinking water at 0, 800, 1,600 or 3,200 ppm (w/w) for 24 hr/day, 7 days/week and 4 weeks. Both the combined inhalation and oral exposures and the single-route exposure through inhalation or ingestion induced centrilobular hypertrophy and single-cell necrosis of hepatocytes, increased plasma levels of alanine aminotransferase (ALT), increased percentage of proliferating cell nuclear antigen (PCNA)-positive hepatocytes without glutathione-*S*-transferase placental form (GST-P)-positive liver foci, and increased relative liver weight. Those hepatic parameters of the DMF-induced effects were classified into hypertrophic, necrotic and proliferative responses according to the pathological characteristics of affected liver. While magnitudes of the hypertrophic and necrotic responses were linearly increased with an increase in amounts of DMF uptake in the single-route exposure groups, those dose-response relationships tended to level off in the combined-exposure groups. Saturation of the hypertrophic and necrotic responses at high dose levels might be attributed to suppression of the metabolic conversion of DMF to its toxic metabolites. Percentage of PCNA-stained hepatocytes classified as the proliferative response was increased more steeply in the combined-exposure groups than in the single-route exposure groups. It was suggested that the proliferative response of hepatocytes to the combined exposures would be greater than that which would be expected under an assumption of additivity for the component proliferative responses to the single-route exposures through inhalation and ingestion.

Key words: Dimethylformamide, Rat, Combined exposure, Hepatotoxicity, Proliferating Cell Nuclear Antigen (PCNA)

INTRODUCTION

N,N-Dimethylformamide (DMF) has been widely used as an organic solvent for synthetic textiles, leathers and polymers such as polyurethane and polyacrylonitrile, and as an intermediate in chemical manufacturing and pharmaceutical industries (IPCS, 1991). The annual production and importation of DMF in Japan were reported to range between 10,000 and 100,000 tons in 2004 (Japan Ministry of Economy, Trade and Industry, 2007). According to the Pollutant Release and Transfer Register (PRTR) Report from the Japan Ministry of the Environment (2007), 4,000 and 400 tons of DMF were released annually into the atmosphere and public waters, respectively, from various sectors such as chemical, rubber, leather and plastic industries in 2005. The total amount of DMF released into the environment was ranked as the 9th environmental contaminant following toluene, xylene, dichloromethane, ethylbenzene, lead, manganese, arsenic and trichloroethylene. Atmospheric concentrations of DMF at sampling points from various areas of Japan in 2000 were reported to range from 0.01 to 0.62 μg/m^3, while con-

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centrations of DMF in public waters ranged from 0.037 to 1.5 ppb (w/w), according to the report from the Japan Ministry of the Environment (2006). Since the PRTR and environmental data suggest the ubiquitous presence of DMF in urban air and public waters, the general population might 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 to be excessively exposed to high levels of DMF primarily through inhalation and dermal contact (IPCS, 1991).

Medical case reports, epidemiological studies and experimental toxicology studies on health or toxic effects of DMF revealed that DMF primarily affects the liver in humans (Fleming *et al.*, 1990; Wang *et al.*, 1991; Redlich *et al.*, 1990; Fiorito *et al.*, 1997; Nomiyama *et al.*, 2001) and in experimental animals (Lundberg *et al.*, 1981; Craig *et al.*, 1984; Kennedy Jr. *et al.*, 1986; Wang *et al.*, 1999; Chieli *et al.*, 1995; Lynch *et al.*, 2003; Senoh *et al.*, 2003; Malley *et al.*, 1994; Senoh *et al.*, 2004). 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). 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 evidence (1999) suggesting lack of carcinogenicity of DMF in experimental animals. More recently, however, Senoh *et al.* (2004) demonstrated that 2-year inhalation exposure to DMF produces hepatocellular adenomas and carcinomas in rats and mice and hepatoblastomas in mice. Since results from a broad range of *in vitro* and *in vivo* genotoxicity assays have been reported to be consistently negative for DMF (IARC, 1999), it is suggested a nongenotoxic-cytotoxic-proliferative mode of action may operate in DMF-induced hapatocarcinogenesis. The nongenotoxic-cytotoxic-proliferative hypothesis of hepatocarcinogenesis (Butterworth *et al.*, 1992) suggested that hepatocellular death by toxic insult and subsequent regenerative proliferation of hepataocytes play crucial roles in an early stage participating in chemically-induced hepatocarcinogenesis. Indeed, it has been recognized that 13-week inhalation exposure of rats and mice to DMF vapor induces such related lesions as necrosis, fragmented or enlarged nuclei and increased mitotic figure in the liver (Lynch *et al.*, 2003; Senoh *et al.*, 2003).

It is important to understand whether exposure of a general population to low levels of ubiquitously present DMF through both inhalation and ingestion may cause any subtle but untoward outcome on the liver, since the toxic effects have been evidenced in the experimental animals exposed to far high levels of DMF through single-route exposure of either inhalation or ingestion (Lundberg *et al.*, 1981; Craig *et al.*, 1984; Kennedy Jr. *et al.*, 1986; Wang *et al.*, 1999; Chieli *et al.*, 1995; Lynch *et al.*, 2003; Senoh *et al.*, 2003; Malley *et al.*, 1994; Senoh *et al.*, 2004). Thus the present study was designed to examine hepatotoxicity induced by combined inhalation and oral exposures of male rats to DMF in comparison with those by single-route exposure through either inhalation or ingestion. Male rats were exposed by inhalation to DMF vapor at 0, 200 or 400 ppm (v/v) for 4 weeks as the inhalation-alone groups, and each of the inhalation groups received oral administration of DMF-formulated drinking water at 0, 800, 1,600 or 3,200 ppm (w/w). Various hepatic parameters of the DMF-induced effects obtained by methodologies of blood biochemistry, histopathology and immunohistochemistry were classified into three different groups of hypertrophic, necrotic and proliferative responses according to pathological characteristics of affected liver (Popp and Cattley, 1991). Dose-response relationships for these three different groups of the responses were compared for additivity of the response magnitudes between the combined-exposure groups and the single-route exposure groups through inhalation and ingestion. We report here on enhanced hepatocellular proliferation by the combined inhalation and oral exposures to DMF in a more than additive manner as expected from the single-route exposures through inhalation and ingestion.

MATERIALS AND METHODS

Chemicals

DMF (99.5% pure) was obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Gas chromatographic analysis showed that no peaks corresponding to impurities were detected either in the 400 ppm (v/v) DMF-containing air in inhalation exposure chambers or in the 3,200 ppm (w/w) DMF-formulated drinking water.

Animals

Four-week-old male F344/DuCrlCrj rats (SPF) were purchased from Charles River Japan, Inc (Atsugi, Japan). The experiment was started when the rats were 6-week-old, after 1-week quarantine and 1-week acclimation. The rats were divided by stratified randomization into 12 body weight-matched groups, each comprising 5 rats. The rats were housed individually in stainless steel wire hanging cages (150 mm W x 216 mm D x 176 mm H) in an inhalation exposure chamber maintained at a temperature of 20 to 24°C and a relative humidity of 30 to 70%, with 12 air changes/hr throughout the 4-week exposure period. An inner volume of the exposure chamber was 1.06 m^3. Four inhalation exposure chambers were installed in

a barrier-system animal room. Fluorescent lighting was controlled automatically to give a 12-hr light/dark cycle. The rats had free access to a gamma-irradiation-sterilized commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan). 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).

Exposure to DMF

The techniques for generating the DMF vapor-air mixture and the system for the combined inhalation and oral exposure to DMF were described in detail in the previous paper (Kano *et al.*, 2002). A group of 5 male rats was exposed by inhalation to DMF vapor at 0 (control), 200 or 400 ppm (v/v) for 6 hr/day, 5 days/week and 4 weeks, and each inhalation group received DMF-formulated water at 0 (control), 800, 1,600 or 3,200 ppm (w/w) for 24 hr/day and 7 days/week for 4 weeks. A total of 12 different groups consisting of 6 combined-exposure, 3 oral-alone and 2 inhalation-alone groups and one untreated control group was used in the present study. The study design of the exposure combinations and target concentrations of DMF in the exposure chamber and in the drinking water are described in Table 1. DMF-formulated or vehicle drinking water was prepared once a week. Exposure-chamber and drinking-water concentrations of DMF were determined by gas chromatography, and those observed concentrations are presented in Table 1.

Clinical observations, analysis and pathological examinations

Rats were observed daily for clinical signs and mortality. Body weights and food and water consumption were measured once a week. All rats surviving to the end of the 4-week exposure period underwent complete necropsy. For blood biochemistry, the surviving animals were bled under ether anesthesia after they were overnight fasting, for terminal necropsy. The blood samples were analyzed with an automatic analyzer (Hitachi 7070: Hitachi, Ltd., Tokyo, Japan) for blood biochemistry. Organs were removed, weighed and examined for macroscopic lesions at necropsy. The organs and tissues were fixed in 10% neutral buffered formalin, and embedded in paraffin for microscopic examination. All tissues were cut into 5 μm-thick sections and stained with hematoxylin and eosin (H & E). The microscopically examined lesions of centrilobular hypertrophy and single-cell necrosis were scored into 4 different grades of severity, ie., 1+: slight, 2+: moderate, 3+: marked and 4+: severe. The averaged severity grade in each group was calculated with the following equation: Σ (grade × number of animals with grade) / number of animals examined.

Proliferation of hepatocytes was determined with slight modification of the widely used methods (Greenwell *et al.*, 1991; Foley *et al.*, 1993) for proliferating cell nuclear antigen (PCNA) immunohistochemical

Table 1. Study Design and observed concentrations of *N,N*-Dimethylformamide (DMF) in Inhalation Chamber and 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	-	-
Inh-0 + Orl-800 ppm	0	800	-	802 ± 14
Inh-0 + Orl-1600 ppm	0	1600	-	1610 ± 20
Inh-0 + Orl-3200 ppm	0	3200	-	3210 ± 32
Inh-200 + Orl-0 ppm	200	0	199.7 ± 3.2	-
Inh-200 + Orl-800 ppm	200	800	199.7 ± 3.2	794 ± 6
Inh-200 + Orl-1600 ppm	200	1600	199.7 ± 3.2	1570 ± 23
Inh-200 + Orl-3200 ppm	200	3200	199.7 ± 3.2	3270 ± 27
Inh-400 + Orl-0 ppm	400	0	399.1 ± 6.8	-
Inh-400 + Orl-800 ppm	400	800	399.1 ± 6.8	798 ± 7
Inh-400 + Orl-1600 ppm	400	1600	399.1 ± 6.8	1600 ± 8
Inh-400 + Orl-3200 ppm	400	3200	399.1 ± 6.8	3250 ± 91

staining. Briefly, paraffin-embedded liver samples sectioned at 5 μm were stained with a mouse monoclonal antibody, using EnVision+ (EV+, Dako, Copenhagen, Denmark) of two-layer dextran polymer visualization system. Tissue sections were counterstained with hematoxylin for detection of PCNA-negative nuclei. The number of hepatocytes with PCNA-positive nuclei and the total number of hepatocytes were counted in three random microscopic fields (x100 magnification) per section. The proliferation index was calculated by dividing the number of hepatocytes with PCNA-positive nuclei by the total number of hepatocytes per section. Results are expressed as means ± S.D. of the total number of sections. Additionally, the livers of all animals were sectioned for further examination of enzyme-altered hepatocellular foci by immunohistochemical staining with antibody of glutathione *S*-transferase placental form (GST-P) (Sato *et al.*, 1984; Satoh *et al.*, 1985; 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, 1988). Polyclonal anti-GST-P antibody was obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). A GST-P-positive liver focus was defined as a focal population of 50 or more hepatocytes which were homogeneously stained brown and were clearly distinguishable from the surrounding normal liver tissue.

Statistical analysis

Statistical comparison was performed between the following groups: a) each of all DMF-exposed groups vs. the untreated control group; b) each of the two combined-exposure groups vs. the oral-alone group; and c) each of the three combined-exposure groups vs. each of the inhalation-alone groups with matching concentrations. Dunnett's test was used for body weights, organ weights and blood biochemistry data. Two-tailed test was used for all statistics, and in all cases, p-value of 0.05 was used as the level of significance.

RESULTS

No death occurred in any of the inhalation-alone, oral-alone and combined-exposure groups during the 4-week exposure period. Terminal body weight significantly decreased in the four combined-exposure groups (Inh-200 + Orl-3,200 ppm, Inh-400 + Orl-400 ppm, Inh-400 + Orl-1,600 ppm and Inh-400 + Orl-3,200 ppm) as compared with the respective controls (Table 2).

Time-averaged water consumption decreased in the four combined-exposure groups (Inh-200 + Orl-1,600 ppm, Inh-200 + Orl-3,200 ppm, Inh-400 + Orl-1,600 ppm

Table 2. Terminal Body Weight, Daily Water Consumption and Estimated Amount of *N,N*-Dimethylformamide (DMF) Uptake

Group Name	Terminal body weight (g) (mean ± S.D.)	Time-averaged water consumption (g/day/rat)	Estimated amount of DMF uptake (mg/kg/day)		
			Inhalation	Drinking water	Total
Inh-0 + Orl-0 ppm	205 ± 4	18.8	0	0	0
Inh-0 + Orl-800 ppm	215 ± 9	20.7	0	83	83
Inh-0 + Orl-1600 ppm	209 ± 5	17.1	0	144	144
Inh-0 + Orl-3200 ppm	192 ± 8	16.1	0	292	292
Inh-200 + Orl-0 ppm	212 ± 11	20.1	121	0	121
Inh-200 + Orl-800 ppm	207 ± 8	19.2	121	80	201
Inh-200 + Orl-1600 ppm	198 ± 11	16.1	121	143	264
Inh-200 + Orl-3200 ppm	181 ± 2 [a,b,c]	14.8	121	287	408
Inh-400 + Orl-0 ppm	193 ± 9	16.4	242	0	242
Inh-400 + Orl-800 ppm	193 ± 10 [c]	17.4	242	77	319
Inh-400 + Orl-1600 ppm	175 ± 6 [a,b,c]	15.4	242	150	392
Inh-400 + Orl-3200 ppm	167 ± 7 [a,b,c]	15.7	242	313	555

Note. a, b and c : Significantly different from untreated control group (Inh-0 + Orl-0 ppm), each inhalation-alone group (Inh-200 + Orl-0, Inh-400 + Orl-0) with matching concentrations and each oral-alone group (Inh-0 + Orl-800, Inh-0 + Orl-1600, Inh-0 + Orl-3200) with matching concentrations, respectively, at $p < 0.05$ by Dunnett test.

and Inh-400 + Orl-3,200 ppm) and in the oral-alone group (Inh-0 + 3,200 ppm), as compared with the untreated control group (Table 2).

Table 2 also shows the estimated amounts of DMF uptake in the inhalation-alone, oral-alone and combined-exposure groups. The calculation of DMF uptake was based on the assumption of the minute volume to be 561 ml/min/kg body weight for rats (Mauderly *et al.*, 1979) and absorption ratios of DMF in the upper and lower respiratory tracts and gastrointestinal tract as 100%. The total amount of DMF uptake was found to decrease in the following order, regardless of the exposure routes: Inh-400 + Orl-3,200 ppm > Inh-200 + Orl-3,200 ppm ≒ Inh-400 + Orl-1,600 ppm > Inh-400 + Orl-800 ppm > Inh-0 + Orl-3,200 ppm > Inh-200 + Orl-1,600 ppm > Inh-400 + Orl-0 ppm > Inh-200 + Orl-800 ppm > Inh-0 + orl-1,600 ppm > Inh-200 + Orl-0 ppm > Inh-0 + Orl-800 ppm.

Relative liver weight significantly increased in all DMF-treated groups except the Inh-200 + Orl-0 ppm and Inh-200 + Orl-800 ppm groups, as compared with the untreated control group (Table 3). Both single-route exposure groups through inhalation and ingestion exhibited a clearly positive dose-response relationship between the estimated amounts of DMF uptake and increased relative liver weight. However, no positive dose-response relationship for increased liver weight could be found in the combined-exposure groups.

Table 3 shows comparison in the DMF-induced effects for five hepatic parameters among the inhalation-alone, oral-alone and combined-exposure groups. Incidences of centrilobular hypertrophy of hepatocytes, which was classified into the hypertrophic response, were increased in all the DMF-treated groups as compared with the untreated control group. A dose-response relationship for hypertrophic response was examined using a linear regression equation between the estimated amounts of DMF uptake and averaged severity grades of centrilobular hypertrophy. The slope of the linear regression equation for the hypertrophic response was steeper in the single-route exposures through inhalation and ingestion than in the combined-exposure groups, indicating that the hypertrophic response tended to level off in the combined-exposure groups as compared with the single-route exposure groups.

Cytolytic release of alanine aminotransferase (ALT) into plasma and single cell necrosis, both of which were classified into the necrotic response, exhibited an essentially similar pattern of the dose-response relationships to that for the hypertrophic response. The slopes of two linear regression equations between estimated amounts of DMF uptake and averaged severity grades of single-cell necrosis and the plasma levels of ALT were steeper in the single-route exposure groups than in the combined-exposure groups (Table 3). A dose-dependent increase in averaged severity grades of single-cell necrosis and the plasma levels of ALT tended to level off in the combined-exposure groups as compared with the single-route exposure groups.

The increased proliferation index of PCNA-positive hepatocytes (Fig. 1) expressed as the percent hepatocytes in S phase, which was classified into the proliferating response, was found in all the DMF-treated groups. A dose-response relationship for the percentage of PCNA-stained hepatocytes exhibited a completely different pattern from the hypertrophic or necrotic response. Percentage of PCNA-positive hepatocytes was increased dose-dependently not only in both the single-route exposure groups but also in the combined-exposure groups. The slope of the linear regression equation for the dose-response relationship was steeper in the combined-exposure groups as a whole than in the single-route exposure groups (Table 3). Notably, the proliferation indices of PCNA-stained hepatocytes in the combined exposure groups with inhalation of 200 ppm were greater than sum of the component proliferation indices in the single-route exposure groups through inhalation and ingestion (Fig. 2A). The combined-exposure groups with inhalation of 200 ppm exhibited a steeper slope for the dose-response relationship than did the same combined groups with inhalation of 400 ppm or the oral-alone groups (Fig. 2B). A slope of the dose-response curve for the combined-exposure groups with inhalation of 400 ppm was decreased to that for the oral-alone group.

It was noteworthy in the present study that neither the GST-P-positive liver foci nor the altered hepatocellular foci stained with H & E could be detected in either the inhalation-alone, oral-alone or combined-exposure groups.

DISCUSSION

It was found in the present study that the combined inhalation and oral exposures of male rats to DMF for 4 weeks induce centrilobular hypertrophy and single cell necrosis of hepatocytes, increased plasma levels of ALT and increased percentage of PCNA-positive hepatocytes without either the GST-P-positive hepatocellular foci or the altered hepatocellular foci stained with H & E, in addition to the increased relative liver weight. Hepatotoxicity induced by the combined exposures was also found to be qualitatively similar to that by the single route exposures through inhalation and ingestion. The present results are consistent with findings of the previ-

Table 3. Changes in hepatic parameteres following combined inhalation and oral exposures or single-route exposures to DMF in male rats.

Group name	No. of animals examined	liver weight(%) (mean ± S.D.)	Centrilobular hypertrophy		Single-cell necrosis		ALT (IU/L) (mean ± S.D.)	PCNA positive hepatocytes(%) (mean ± S.D.)
			Incidence (%)	(Averaged severity)	Incidence (%)	(Averaged severity)		
Inh-0 + Orl-0 ppm	5	3.10 ± 0.05	0	(0)	0	(0)	35 ± 1	0.3 ± 0.1
Inh-0 + Orl-800 ppm	5	4.08 ± 0.17 [a]	100	(1.0)	60	(0.6)	51 ± 10	1.0 ± 0.5
Inh-0 + Orl-1600 ppm	5	4.11 ± 0.09 [a]	80	(0.8)	80	(1.0)	53 ± 7	1.6 ± 0.6 [a]
Inh-0 + Orl-3200 ppm	5	4.23 ± 0.21 [a]	100	(1.0)	100	(1.8)	76 ± 15 [a]	2.6 ± 1.8 [a]
Inh-200 + Orl-0 ppm	5	3.74 ± 0.13	40	(0.4)	100	(1.4)	60 ± 12 [a]	0.6 ± 0.2 [a]
Inh-200 + Orl-800 ppm	5	3.93 ± 0.16	100	(1.2)	100	(2.0)	88 ± 14 [a]	1.9 ± 0.6 [a,b]
Inh-200 + Orl-1600 ppm	5	4.01 ± 0.36 [a]	100	(1.6)	100	(2.0)	93 ± 26 [a,b]	3.6 ± 2.4 [a,b]
Inh-200 + Orl-3200 ppm	5	3.97 ± 0.11 [a]	100	(1.8)	100	(2.4)	97 ± 20 [a,b]	5.8 ± 1.5 [a,b,c]
Inh-400 + Orl-0 ppm	5	4.03 ± 0.12 [a]	100	(2.0)	100	(2.0)	122 ± 27 [a]	1.4 ± 0.7 [a]
Inh-400 + Orl-800 ppm	5	4.10 ± 0.04 [a]	100	(1.8)	100	(2.8)	85 ± 17 [a,c]	2.6 ± 1.0 [a,c]
Inh-400 + Orl-1600 ppm	5	3.98 ± 0.19 [a]	100	(2.0)	100	(2.0)	95 ± 21 [a,c]	3.6 ± 2.0 [a]
Inh-400 + Orl-3200 ppm	5	4.07 ± 0.17 [a]	100	(2.0)	100	(2.4)	134 ± 53 [a,c]	4.4 ± 1.9 [a,b]
DMF single-route exposure groups								
Regression equation			*y = 0.0046x + 0.1942*		*y = 0.0066x + 0.1613*		*y = 0.221x + 33.719*	*y = 0.0068x + 0.2564*
DMF combined-exposure groups								
Regression equation			*y=0.0037x + 0.3574*		*y = 0.0041x + 0.6926*		*y = 0.1542x + 42.322*	*y = 0.0086x + 0.5523*

Note1. a, b and c : Significantly different from untreated control group (Inh-0 + Orl-0 ppm), each inhalation-alone group (Inh-200 + Orl-0, Inh-400 + Orl-0) with matching concentrations and each oral-alone group (Inh-0 + Orl-800, Inh-0 + Orl-1600, Inh-0 + Orl-3200) with matching concentrations, respectively, at $p < 0.05$ by Dunnett test.

Note2. PCNA : Proliferating cell nuclear antigen

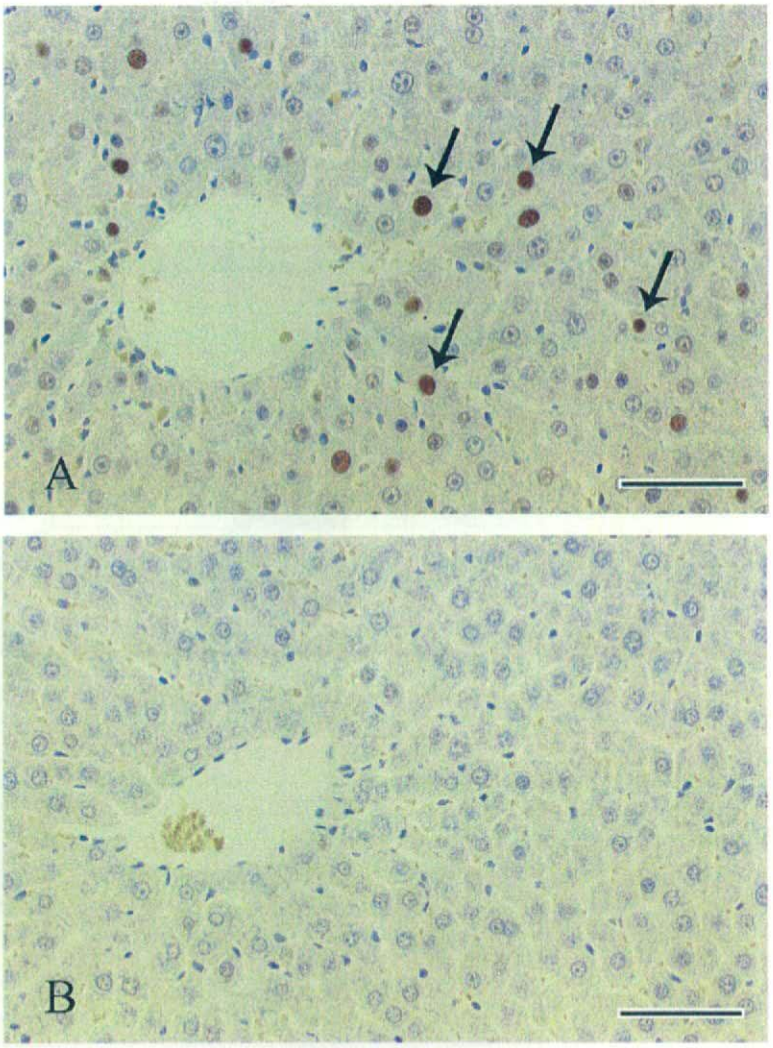

Fig. 1. A photomicrograph of a PCNA immunohistochemically stained liver from a male rat receiving the Inh-200 + Orl-3,200 ppm DMF for 4 weeks (A) and another photomicrograph of the liver showing absence of PCNA-stained hepatocytes in an untreated rat (B). PCNA-labeled hepatocytes are easily recognized (arrows). Bar indicates 100 μm.

ously reported experimental studies that repeated inhalation exposure to and oral administration of DMF for up to 13 weeks induce centrilobular hypertrophy and necrosis of hepatocytes and increased cytolytic release of ALT and aspartate transaminase (AST) into plasma (Lundberg *et al.*, 1981; Craig *et al.*, 1984; Kennedy Jr. *et al.*, 1986; Wang *et al.*, 1999; Chieli *et al.*, 1995; Lynch *et al.*, 2003, Senoh *et al.*, 2003). In addition, some of these studies demonstrated that a proliferative lesion including increased mitotic figure occurs in the liver of rats exposed to DMF (Lynch *et al.*, 2003; Senoh *et al.*, 2003). In the present study, the hepatic parameters of the DMF-induced effects were classified into the three different groups of hypertrophic, necrotic and proliferative responses according to the pathological characteristics of affected liver (Popp and Cattley, 1991). This classification allowed plausible elucidation of differences in the dose-response relationship between the combined-exposure groups and the single-route exposure groups, which would shed light on the early hepatocellular events participating in the

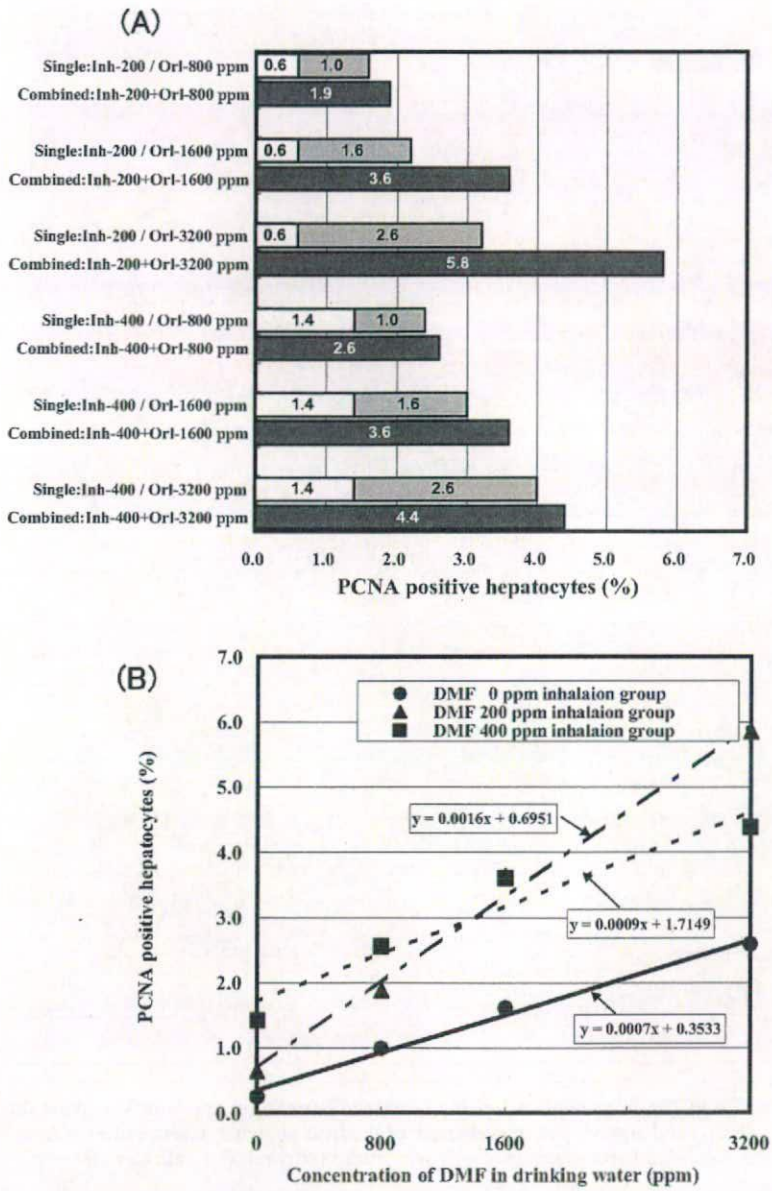

Fig. 2. (A): Comparison of proliferation indices of PCNA-staind hepatocytes in combined exposure groups (solid bars) with the sum of component proliferation indices in single-route exposure groups through inhalation (open bars) and ingestion (shaded bars). (B): Averaged percentage of PCNA-positive hepatocytes in rats treated with DMF at a route of inhalation exposure as a function of drinking-water concentrations of DMF.

chemically induced hepatocarcinogenesis. DMF is recognized as a nongenotoxic agent in various *in vitro* and *in vivo* genotoxicity assays (IARC, 1999). Nongenotoxic agents are reported to exhibit carcinogenic activity at doses that also produce cytolethality and regenerative cell proliferation according to a nongenotoxic-cytotoxic-proliferative hypothesis (Butterworth *et al.*, 1992). Therefore, the necrotic and proliferative responses of hepatocytes as indicated by single-cell necrosis, increased plasma levels of ALT and increased percentage of PCNA-

positive hepatocytes found in the present study are considered to play crucially important roles in early stages of development of DMF-induced liver tumors, since Senoh *et al.* (2003 and 2004) demonstrated induction of hepatocellular tumors in rats and mice exposed by inhalation to DMF for 2 years through hepatic necrosis and increased mitotic figure, both of which occurred earlier in the animals exposed to DMF for 13 weeks.

It is noteworthy in the present study that some quantitative differences in the dose-response relationship for the hypertrophic, necrotic and proliferative responses are found between the combined-exposure groups and the single-route exposure groups. Response magnitudes of single-cell necrosis and plasma ALT were increased linearly with an increase in estimated amounts of DMF uptake in the single-route exposure groups, but tended to level off with a further increase in the amounts of DMF uptake in the combined-exposure groups. The suppressive tendency of the necrotic responses with a further increase in amounts of DMF uptake is consistent with Lundberg *et al.*'s finding (1986) that high doses of DMF inhibited liver damage as measured by serum levels of sorbitol dehydrogenese in rats, and thus seems to be accounted for in terms of saturated DMF metabolism and limited production of potently hepatotoxic metabolites. It has been reported that DMF is metabolized to *N*-(hydroxymethyl)-*N*-methylformamide (HMMF) and then to *N*-methylformamide (NMF), and finally is conjugated with GST to form *N*-acetyl-*S*-(*N*-methylcarbamoyl)-*N*-methylformamide (AMCC) which is excreted into urine (Lundberg *et al.*, 1986; Chieli *et al.*, 1995). Hundley *et al.* (1993) reported that inhalation exposure to 500 ppm DMF saturates the metabolic conversion of DMF to AMCC, resulting in a disproportionate increase in plasma levels of DMF. Mráz *et al.* (1989, 1993) demonstrated that P450 2E1 is responsible for the catalysis of DMF metabolism and that DMF inhibits the P450 2E1-mediated metabolism of its own metabolites. It has been reported that that NMF is potently hepatotoxic as compared with DMF or HMMF, and that the most toxic intermediate metabolite, methylisocyanate, might be generated from NMF (Kestell *et al.*, 1987; Gescher, 1993). Therefore, it is suggested that increased amounts of DMF uptake in the combined-exposure groups cause a disproportionate increase in blood levels of DMF that would then suppress metabolic conversion of DMF to AMCC, presumably resulting in decreased production of the two potently hepatotoxic metabolites, NMF and methylisocyanate.

On the other hand, percentage of PCNA-positive hepatocytes as the proliferative response was found to exhibit a different pattern of the dose-response relationship throughout the entire range of the total amounts of DMF uptake. In sharp contrast to the dose-response relationships for hypertrophic and necrotic responses, the linear regression equation of the dose-response relationship for the percentage of PCNA-stained hepatocytes was steeper in the combined-exposure groups as a whole than in the single-route exposure groups. Especially, the combined-exposure groups with inhalation of 200 ppm DMF exhibited the steepest slope among the three different exposure groups. Thus, the present finding suggested that the proliferative response of hepatocytes to the combined exposures still remains enhancing, while the necrotic response tended to level off presumably resulting from suppression of the metabolic conversion of DMF to the toxic metabolites, and that these differential effects are clearly seen in the combined exposure groups with inhalation of 200 ppm.

It was noteworthy in the present study that the increased proliferation indices of PCNA-stained hepatocytes in the combined exposure groups are greater than the sum of the component proliferation indices in the single-route exposure groups through inhalation and ingestion, and that the slope of the linear dose-response relationship for the proliferation indices in the combined-exposure groups with inhalation of 200 ppm DMF was steeper than that in the single-route exposure groups through inhalation and ingestion. The USA Environmental Protection Agency (2000) proposed the definitions of additivity and synergism for the effects of chemical mixtures. Additivity was defined as the effect of the combination being equal to the sum of the effect of the individual chemicals, while synergism was defined as the effect of the combination being greater than that suggested by the component toxic effects of individual chemicals. Application of the USA. EPA's definition of additivity and synergism to the present results of the combined exposures suggested that the proliferative response of hepatocytes to the combined exposures to DMF would be greater than that which would be expected under an assumption that the component proliferative responses to the single-route exposures through inhalation and ingestion are additive (i.e., synergistic).

The PCNA immunohistochemical staining has been used for evaluating the capability of the liver to regenerate in response to hepatocellular death following hepatotoxic insult such as carbon tetrachloride (CCl_4). Monticello *et al.* (1995) demonstrated that a single necrogenic dose of CCl_4 at 120 mg/kg increases a plasma level of ALT from 59 to 2,492 IU/l and percent of hepatocytes in S phase from 1% to 20%. The difference in necrotic and proliferative potentials between CCl_4 and DMF can be

seen in absence of both the GST-P-positive foci found in the present study and the altered hepatocellular foci following 13-week inhalation exposure of rats to DMF vapor (Senoh *et al.*, 2003), whereas 13-week inhalation exposure to CCl_4 vapor induced both the GST-P-positive hepatocellular foci and the acidophilic, basophilic and clear altered cell foci (Nagano *et al.*, 2007a). Necrotic, proliferative and tumor-initiating potentials of DMF are thought to be less potent than those of CCl_4.

On the other hand, it is of interest to note that neither the GST-P-positive liver foci nor the altered hepatocellular foci stained with hematoxylin and eosin could be detected in any of the DMF-treated rats, while the percentage of PCNA-stained hepatocytes was increased dose-dependently among the combined-exposure, inhalation-alone and oral-alone groups. GST-P is known as a good specific marker enzyme for detecting an early histogenetic stage participating to the development of rat hepatocellular tumors by chemical carcinogens (Sato *et al.*, 1984; Kitahara *et al.*, 1984; Satoh *et al.*, 1985; Tatematsu *et al.*, 1985; Ito *et al.*, 1988). Ito *et al.* (1988; 1997; 2000) reported that almost all hepatocarcinogens induced the GST-P-positive liver foci which allowed prediction of hepatocarcinogenicity with high probability. The absence of the GST-P-positive liver foci in the DMF-treated male rats suggests that the 4-week treatment with DMF enhances the regenerative proliferation of hepatocytes in response to liver injury, but does not permit to develop to more advanced stage of formation of the GST-P-positive liver foci which would be characterized by focal and clonal proliferation of hepatocytes and be indicative of the tumor initiation.

In conclusion, the hypertrophic and necrotic responses tended to level off in the combined-exposure groups as compared with those in the single-route exposures. However, the proliferative response as indicated by the increased percentage of PCNA-stained hepatocytes was enhanced in the combined-exposure groups, especially in those with inhalation of 200 ppm. It was suggested that the combined inhalation and oral exposures would enhance the hepatocellular proliferation in a more than additive manner (i.e., synergistically) expected from the single-route exposures through inhalation and ingestion. In consideration of the CCl_4-induced GST-P-positive hepatocellular foci and hepatocarcinogenicity (Nagano *et al.*, 2007a and b) as well as the DMF-induced liver tumors (Senoh *et al.*, 2004), the proliferative responses of hepatocytes to the combined inhalation and oral exposures to DMF would play a facilitatory role in formation of the enzyme-altered hepatocellular foci and hepatocellular tumors.

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Original Article

Occurrence of Two Different Types of Glutathione *S*-Transferase Placental Form-Positive Hepatocytes after a Single Administration of 2,3,7,8-Tetrabromodibenzo-*p*-dioxin in Rats

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Abstract: Occurrence of glutathione *S*-transferase placental form (GST-P)-positive hepatocytes was examined, using 15 Wistar rats of both sexes each orally administered 2,3,7,8-tetrabromodibenzo-*p*-dioxin (TBDD) by gavage at a single dose of 0, 10, 30, 100 or 300 μg/kg body weight. Liver tissues were stained with anti-GST-P antibody. Two different types of GST-P-positive hepatocytes were found in the TBDD-dosed rat. One type was of the hepatocytes stained homogeneously with anti-GST-P antibody and clearly distinguishable from the surrounding normal tissue. The foci were composed of 2 to 60 hepatocytes exhibiting morphologically focal and clonal proliferation. The GST-P-positive hepatocellular foci occurred at two higher dose levels and only on Day 36 after the single administration. Another type was of the area occupied by the positively but heterogeneously stained hepatocytes appearing predominantly in the centrilobular region, at lower dose levels and persistently on Day 2 through 36. The stained hepatocytes appeared to be neither focally nor clonally proliferating. Females were more susceptible to formation of the two differently stained hepatocytes than males. It is suggested that the GST-P-positive foci represent an early stage of hepatocarcinogenesis, while the GST-P-positive area is associated with the induction of detoxifying Phase II GSTs.

Key words: Glutathione *S*-transferase placental form (GST-P), Enzyme-altered cell foci, Hepatocarcinogenicity, Rat, 2,3,7,8-Tetrabromodibenzo-*p*-dioxin

Introduction

Polybrominated aromatic compounds including decabromodiphenylether and tetrabromobisphenol have been widely used in plastics, carpets, textiles and other polymer products as flame retardants. Polybromodibenzo-*p*-dioxins (PBDDs) and polybromodibenzofurans (PBDFs) are inadvertently formed in thermal reactions from the polymers containing brominated flame retardants (BFRs)[1]. The formation of toxic compounds from incineration, accidental burning and fires[1–4] of BFR-containing polymers and from the extrusion and molding processes[1, 5, 6] in the manufacture of these BFR products has raised much concern about health risks for the public and workers. Waste residues from various BFR products incinerated in a laboratory-scale incinerator were reported to contain total PBDDs/PBDFs ranging between 3,000 and 130,000 ng/g[4]. Workplace air concentrations of tetra-BDDs and tetra-BDFs measured during the

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extruder production and injection molding of polybutyleneterephthalate polymer blended with glass fiber, Sb_2O_3 and decabromodiphenylether were reported to be 2 and 34 ng/m^3, respectively[5]. The same manufacturing process using tetrabromobisphenol was reported to contaminate workplace air with total PBDD/PBDF ranging from 30 to 260 pg/m^3 [6]. Atmospheric concentrations of total PBDDs/PBDFs in Kyoto City were reported to range between 1.1 and 11 pg/m^3 in the gaseous state and between 0.38 and 1.2 pg/m^3 in particulates in 2000 and 2001[7]. Adipose tissue levels of 2,3,7,8-tetrabromodibenzo-*p*-dioxin (TBDD) in humans from the general Japanese population was reported to be a median value of 1.7 pg/g with a range of 0.8–4.2 pg/g in 1970 and a median value of 0.5 pg/g with a range of 0.1–2.0 pg/g in 2000[8].

Little is known about toxicity of PBDD and PBDF, as compared with vast knowledge on toxicity of the chlorinated analogues[9]. Except for a medical case report[10] on laboratory exposure of a chemist to TBDD, there are few medical case reports and epidemiological studies or animal toxicology studies of PBDD/PBDF available for human health risk assessments. Experimental toxicology studies have revealed that TBDD caused systemic, hematological, hepatic, teratogenic and myelogenic toxicities[11–15].

We previously reported that a single oral administration of TBDD by gavage in rats induced necrosis, multinucleated and disarranged hepatocytes, hepatocellular hypertrophy and tigroid cytoplasmic basophilia, in addition to increased hepatic levels of aryl hydrocarbon hydroxylase (AHH), ethoxycoumarin *O*-deethylase (ECOD) and ethoxyresorufin *O*-deethylase (EROD)[15]. We also suggested that the acute hepatotoxicity of TBDD was as potent as that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[15]. Two 2-yr bioassay studies[16, 17] have demonstrated positive hepatocarcinogenicity of TCDD in the female rats orally administered TCDD, and two-stage model studies[18–21] for hepatocarcinogenesis have classified TCDD as a tumor promoter. Similarities in molecular structure and acute hepatotoxicity between TBDD and TCDD led us to suggest that there exists any early detectable stage participating to possible development of the liver tumors induced by administration of TBDD to rats, since multinucleated and disarranged hepatocytes found in the previous study[15] of acute TBDD hepatotoxicity are causally related to development of liver tumors. Glutathione *S*-transferase-placental form (GST-P) is known as a good specific marker enzyme for detecting an early histogenetic stage participating to the development of rat hepatocellular tumors by chemical carcinogens[22–30]. Ito *et al.*[24, 29, 30] reported that almost all hepatocarcinogens induced the GST-P-positive liver foci which allowed prediction of hepatocarcinogenicity with high probability. Besides, glutathione *S*-transferases (GSTs) which are known as Phase II detoxifying liver enzymes have been reported to be induced in human hepatocyte cultures by TCDD[31] and in rat liver parenchymal cells by polychlorinated biphenyl having TCDD-like coplanar structure[32, 33]. Thus, immunohistochemical staining of liver sections with anti-GST-P antibody is thought to serve as a good tool for detecting not only an early histogenetic stage participating to possible development of hepatocarcinogenicity but also induction of Phase II detoxifying GSTs in the hepatocytes of TBDD-dosed rat.

The present study was designed to explore an early detectable stage participating to the formation of preneoplastic enzyme-altered hepatocellular foci in the TBDD-dosed rat, using an immunohistochemical method with anti-GST-P antibody. Besides, the immunohistochemical staining of livers with the anti-GST-P antibody was examined for detecting induction of GST enzymes in the hepatocytes of TBDD-dosed rat. We examined dose- and time-related occurrence of two different kinds of GST-P-positive hepatocytes, the GST-P-positive liver foci exhibiting focal and clonal proliferation and the area occupied by the heterogeneously GST-P-stained hepatocytes without appearance of focal or clonal proliferation. These GST-P-positive hepatocytes were discussed in light of possible development of liver tumors and induction of hepatic GST enzymes by TBDD.

Materials and Methods

Chemicals

TBDD (purity >98%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). TBDD was predissolved in degassed toluene and added to corn oil. The toluene concentration in corn oil was 5% for all treated groups. Polyclonal antibody of GST-P was obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

Animals

Crj:Wistar rats of both sexes were purchased at the age of 4 wk from Charles River Japan, Inc (Tsukuba, Japan). The animals were quarantined and acclimated for 2 wk, and then divided by stratified randomization into 5 body weight-matched groups, each comprising 15 rats of both sexes. The conditions under which the animals were raised were described in detail previously[14, 15]. The animals were cared for according to the Guide for the Care and Use of Laboratory Animals[34]. The present study was approved by the ethics committee of the Japan Bioassay Research Center (JBRC).

Experimental design and pathological examination

TBDD was orally administered by gavage to groups of 6-wk-old rats of both sexes each at a dose of 10, 30, 100 or 300 μg/kg body weight. Control groups of both sexes received corn oil containing 5% toluene. The total volume administrated to rats was 5 ml/kg body weight.

Animals surviving to the scheduled necropsy on Days 2, 7 and 36 or found dead during the 36-d observation period underwent complete necropsy. For microscopic examination, liver tissues were fixed in 10% neutral buffered formalin, and were embedded in paraffin. Tissue sections of 5 μm thick were prepared and stained with hematoxylin and eosin for conventional microscopic examination. One section at the median lobe was stained immunohistochemically with anti-GST-P antibody[24–28], using EnVision+ (EV+, Dako, Copenhagen, Denmark) of two-layer dextran polymer visualization system[35]. Focal populations having two or more homogeneously stained and agglomerated hepatocytes were counted as the GST-P-positive foci with a light microscope using an eye-piece grid, and the area of the liver section was measured with a planimeter. Number of the GST-P-positive foci per unit liver area (cm^2) and number of GST-P-positive hepatocytes in each of the foci were determined for each animal. The area which was occupied by the GST-P-positively but heterogeneously stained hepatocytes was defined as the GST-P positive area. Its severity grade was scored as 1+ (slight) for less than 10% of total liver area, 2+ (moderate) for 10 to 40% of the total liver area and 3+ (marked) for 40 to 80% of the total liver area occupied by the GST-P positive area. The averaged severity grade in each group was calculated with a following equation. $\sum$(grade × number of animals with grade) / number of animals examined.

Results

Immunohistochemical examination of the liver tissue using anti-GST-P antibody revealed two different types of GST-P-positive hepatocytes in the TBDD-dosed rats. One type was of the hepatocytes stained homogeneously with anti-GST-P antibody and clearly distinguishable from the surrounding normal tissue. The positively stained hepatocytes exhibited morphologically focal and clonal proliferation. Those foci were not localized in any specific zone of liver lobules. Fig. 1A and the left side of Fig. 1C show the GST-P-positive foci composed of 58 and 10 hepatocytes, respectively. In the liver stained with hematoxylin and eosin, however, any altered hepatocellular foci characterized by the altered cytoplasm according to differently tinctorial properties[36] could not be detected in the present study. On the other hand, another type of the hepatocytes stained heterogeneously with anti-GST-P antibody was found in the TBDD-dosed rat (Fig. 1B and the right side of Fig. 1C). The heterogeneously stained hepatocytes appeared to be neither focally nor clonally proliferating without clearly distinguishable demarcation from surrounding normal hepatocytes, in sharp contrast to the GST-P-positive liver foci. The heterogeneously stained area occupied by the GST-P-positive hepatocytes was present dominantly in the centrilobular region (Fig. 1B and the right side of Fig. 1C). This type of the positively stained hepatocytes was termed as the GST-P-positive area of hepatocytes. Different location and staining of the GST-P-positive area from the GST-P-positive foci in the liver can be illustrated in Fig. 1C. Absence of false-positive staining due to endogenous biotin for the two different kinds of the GST-P-positive hepatocytes was confirmed by staining the liver of the vehicle-dosed control rat with the present staining method using the EnVision+ of two-layer dextran polymer visualization system[35] (Fig. 1D).

The GST-P-positive foci were found in both 100 and 300 μg TBDD/kg-dosed rats of both sexes sacrificed on Day 36 after the single administration (Table 1). Both the number of GST-P-positive foci per unit liver area and the number of GST-P-positive hepatocytes in each of the foci were increased dose-dependently in the TBDD-dosed males but not in the TBDD-dosed females. Apparent lack of the dose-response relationships for these two foci parameters in the 300 μg TBDD/kg-dosed female rats was attributed to deaths of the three rats before Day 36. More foci were found in females than in males, indicating a gender difference in formation of the GST-P-positive foci.

As shown in Table 1, the GST-P-positive area appeared on Day 2 through 36, and was found in the males dosed 30 μg/kg and above and in the females dosed 10 μg/kg and above. Both the number of animals bearing the GST-P-positive area and the averaged severity grades for the GST-P-positive area were increased dose- and time-dependently in both the TBDD-dosed males and females, except for the 300 μg TBDD/kg-dosed females on Day 36 which showed the lack of the dose-response relationships because of the deaths of the three rats before Day 36. Notably, the GST-P-positive area was manifest at lower dose levels and earlier days in the TBDD-dosed rats of both sexes than the GST-P-positive foci. Females were also more susceptible to this type of hepatic lesion than males, indicating a gender difference in the induction of the enzyme-altered area of hepatocytes.

Discussion

It was found in the present study that a single oral administration of TBDD by gavage to male and female Wistar rats induces both the GST-P-positive foci com-