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 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/m³, while con-
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; Chiel 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 hepatocarcinogenesis. The nongenotoxic-cytotoxic-proliferative hypothesis of hepatocarcinogenesis (Butterworth et al., 1992) suggested that hepatocellular death by toxic insult and subsequent regenerative proliferation of hepatocytes 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 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; Chiel 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/DuCrCry 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 m³. 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 Bio-assay 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, i.e., 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

<table>
<thead>
<tr>
<th>Group Name</th>
<th>Target concentration (ppm(v/v))</th>
<th>Observed concentration (mean ± S.D.)</th>
<th>Inhilation (ppm(v/v))</th>
<th>Drinking water (ppm(w/w))</th>
<th>Inhilation (ppm(v/v))</th>
<th>Drinking water (ppm(w/w))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inh-0 + Orl-0 ppm</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Inh-0 + Orl-800 ppm</td>
<td>800</td>
<td>-</td>
<td>0</td>
<td>802 ± 14</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Inh-0 + Orl-1600 ppm</td>
<td>1600</td>
<td>-</td>
<td>0</td>
<td>1610 ± 20</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Inh-0 + Orl-3200 ppm</td>
<td>3200</td>
<td>-</td>
<td>0</td>
<td>3210 ± 32</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Inh-200 + Orl-0 ppm</td>
<td>200</td>
<td>199.7 ± 3.2</td>
<td>200</td>
<td>794 ± 6</td>
<td>200</td>
<td>1570 ± 23</td>
</tr>
<tr>
<td>Inh-200 + Orl-800 ppm</td>
<td>800</td>
<td>199.7 ± 3.2</td>
<td>200</td>
<td>1570 ± 23</td>
<td>200</td>
<td>3270 ± 27</td>
</tr>
<tr>
<td>Inh-200 + Orl-1600 ppm</td>
<td>1600</td>
<td>199.7 ± 3.2</td>
<td>200</td>
<td>3270 ± 27</td>
<td>200</td>
<td>3270 ± 27</td>
</tr>
<tr>
<td>Inh-200 + Orl-3200 ppm</td>
<td>3200</td>
<td>199.7 ± 3.2</td>
<td>200</td>
<td>3270 ± 27</td>
<td>200</td>
<td>3270 ± 27</td>
</tr>
<tr>
<td>Inh-400 + Orl-0 ppm</td>
<td>400</td>
<td>399.1 ± 6.8</td>
<td>400</td>
<td>798 ± 7</td>
<td>400</td>
<td>1600 ± 8</td>
</tr>
<tr>
<td>Inh-400 + Orl-800 ppm</td>
<td>800</td>
<td>399.1 ± 6.8</td>
<td>400</td>
<td>1600 ± 8</td>
<td>400</td>
<td>3250 ± 91</td>
</tr>
<tr>
<td>Inh-400 + Orl-1600 ppm</td>
<td>1600</td>
<td>399.1 ± 6.8</td>
<td>400</td>
<td>3250 ± 91</td>
<td>400</td>
<td>3250 ± 91</td>
</tr>
</tbody>
</table>
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; Tatatematsu 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 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) with matching concentrations and each oral-alone group (Inh-0 + Orl-800, Inh-0 + Orl-1,600, Inh-0 + Orl-3,200) with matching concentrations, respectively, at p < 0.05 by Dunnett test.

<table>
<thead>
<tr>
<th>Group Name</th>
<th>Terminal body weight (g) (mean ± S.D.)</th>
<th>Time-averaged water consumption (g/day/rat)</th>
<th>Estimated amount of DMF uptake (mg/kg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inh-0 + Orl-0 ppm</td>
<td>205 ± 4</td>
<td>18.8</td>
<td>0</td>
</tr>
<tr>
<td>Inh-0 + Orl-800 ppm</td>
<td>215 ± 9</td>
<td>20.7</td>
<td>83</td>
</tr>
<tr>
<td>Inh-0 + Orl-1600 ppm</td>
<td>209 ± 5</td>
<td>17.1</td>
<td>144</td>
</tr>
<tr>
<td>Inh-0 + Orl-3200 ppm</td>
<td>192 ± 8</td>
<td>16.1</td>
<td>292</td>
</tr>
<tr>
<td>Inh-200 + Orl-0 ppm</td>
<td>212 ± 11</td>
<td>20.1</td>
<td>121</td>
</tr>
<tr>
<td>Inh-200 + Orl-800 ppm</td>
<td>207 ± 8</td>
<td>19.2</td>
<td>80</td>
</tr>
<tr>
<td>Inh-200 + Orl-1600 ppm</td>
<td>198 ± 11</td>
<td>16.1</td>
<td>143</td>
</tr>
<tr>
<td>Inh-200 + Orl-3200 ppm</td>
<td>181 ± 2 abcd</td>
<td>14.8</td>
<td>287</td>
</tr>
<tr>
<td>Inh-400 + Orl-0 ppm</td>
<td>193 ± 9</td>
<td>16.4</td>
<td>242</td>
</tr>
<tr>
<td>Inh-400 + Orl-800 ppm</td>
<td>193 ± 10 cabcd</td>
<td>17.4</td>
<td>77</td>
</tr>
<tr>
<td>Inh-400 + Orl-1600 ppm</td>
<td>175 ± 6 abcd</td>
<td>15.4</td>
<td>150</td>
</tr>
<tr>
<td>Inh-400 + Orl-3200 ppm</td>
<td>167 ± 7 abcd</td>
<td>15.7</td>
<td>313</td>
</tr>
</tbody>
</table>

*Note. a, b and c*: Sigificantly 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-1,600, Inh-0 + Orl-3,200) with matching concentrations, respectively, at p < 0.05 by Dunnett test.
Enhanced hepatocellular proliferation of combined exposure to DMF.

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 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 parameters following combined inhalation and oral exposures or single-route exposures to DMF in male rats.

<table>
<thead>
<tr>
<th>Group name</th>
<th>No. of animals examined</th>
<th>Liver weight % (mean ± S.D.)</th>
<th>Centrilobular hypertrophy</th>
<th>Single-cell necrosis</th>
<th>ALT (IU/L) (mean ± S.D.)</th>
<th>PCNA positive hepatocytes % (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inh-0 + Orl-0 ppm</td>
<td>5</td>
<td>3.10 ± 0.05 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>35 ± 1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Inh-0 + Orl-800 ppm</td>
<td>5</td>
<td>4.08 ± 0.17 * (1.0)</td>
<td>100 (1.0)</td>
<td>60 (0.6)</td>
<td>51 ± 10</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Inh-0 + Orl-1600 ppm</td>
<td>5</td>
<td>4.11 ± 0.09 * (0.8)</td>
<td>80 (1.0)</td>
<td>80 (1.0)</td>
<td>53 ± 7</td>
<td>1.6 ± 0.6 *</td>
</tr>
<tr>
<td>Inh-0 + Orl-3200 ppm</td>
<td>5</td>
<td>4.23 ± 0.21 * (1.0)</td>
<td>100 (1.0)</td>
<td>100 (1.8)</td>
<td>76 ± 15 *</td>
<td>2.6 ± 1.8 *</td>
</tr>
<tr>
<td>Inh-200 + Orl-0 ppm</td>
<td>5</td>
<td>3.74 ± 0.13 (0.4)</td>
<td>40 (0.4)</td>
<td>100 (1.4)</td>
<td>60 ± 12 *</td>
<td>0.6 ± 0.2 *</td>
</tr>
<tr>
<td>Inh-200 + Orl-800 ppm</td>
<td>5</td>
<td>3.93 ± 0.16 (1.2)</td>
<td>100 (2.0)</td>
<td>100 (2.0)</td>
<td>88 ± 14 *</td>
<td>1.9 ± 0.6 *</td>
</tr>
<tr>
<td>Inh-200 + Orl-1600 ppm</td>
<td>5</td>
<td>4.01 ± 0.36 * (1.6)</td>
<td>100 (2.0)</td>
<td>100 (2.0)</td>
<td>93 ± 26 *</td>
<td>3.6 ± 2.4 *</td>
</tr>
<tr>
<td>Inh-200 + Orl-3200 ppm</td>
<td>5</td>
<td>3.97 ± 0.11 * (1.8)</td>
<td>100 (2.4)</td>
<td>97 ± 20 *</td>
<td>5.8 ± 1.5 *</td>
<td>3.6 ± 2.0 *</td>
</tr>
<tr>
<td>Inh-400 + Orl-0 ppm</td>
<td>5</td>
<td>4.03 ± 0.12 * (2.0)</td>
<td>100 (2.0)</td>
<td>100 (2.0)</td>
<td>122 ± 27 *</td>
<td>1.4 ± 0.7 *</td>
</tr>
<tr>
<td>Inh-400 + Orl-800 ppm</td>
<td>5</td>
<td>4.10 ± 0.04 * (1.8)</td>
<td>100 (2.8)</td>
<td>100 (2.8)</td>
<td>85 ± 17 *</td>
<td>2.6 ± 1.0 *</td>
</tr>
<tr>
<td>Inh-400 + Orl-1600 ppm</td>
<td>5</td>
<td>3.98 ± 0.19 * (2.0)</td>
<td>100 (2.0)</td>
<td>100 (2.0)</td>
<td>95 ± 21 *</td>
<td>3.6 ± 2.0 *</td>
</tr>
<tr>
<td>Inh-400 + Orl-3200 ppm</td>
<td>5</td>
<td>4.07 ± 0.17 * (2.0)</td>
<td>100 (2.4)</td>
<td>134 ± 53 *</td>
<td>4.4 ± 1.9 *</td>
<td>3.6 ± 2.0 *</td>
</tr>
</tbody>
</table>

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

\[ y = 0.0037x + 0.3574 \]
\[ y = 0.0041x + 0.6926 \]
\[ y = 0.1542x + 42.322 \]
\[ y = 0.0086x + 0.5523 \]

Note 1. 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.

Note 2. PCNA: Proliferating cell nuclear antigen
Previously reported experimental studies have shown that repeated inhalation exposure to and oral administration of DMF for up to 13 weeks induce centrilobular hypertrophy and necrosis of hepatocytes and increased cytolysis 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. 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.
chemically induced hepatocarcinogenesis. DMF is recognized as a nongenotoxic agent in various \textit{in vitro} and \textit{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 \textit{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 as shown in Fig. 2, support the hypothesis of nongenotoxic mechanisms of carcinogenesis.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{(A): Comparison of proliferation indices of PCNA-stained 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.}
\end{figure}
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 dehydrogenase 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 (CCl4). Monti-cello et al. (1995) demonstrated that a single necrogenic dose of CCl4 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 CCl4 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 CCl₄ 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 CCl₄.

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 to be 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 repeated 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 hepatic proliferation in a more than additive manner (i.e., synergistically) expected from the single-route exposures through inhalation and ingestion. In consideration of the high percentage of PCNA-stained hepatocytes in 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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