Chronic Inhalation Toxicity and Carcinogenicity Studies of 3-Chloro-2-methylpropene in BDF1 Mice

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Abstract: Chronic toxicity and carcinogenicity studies of 3-chloro-2-methylpropene (CMP), which has been widely used as an insecticide and chemical intermediate, were carried out in BDF1 mice. CMP was administered to mice in groups of 50 male and 50 female mice by the inhalation route 5 days per week for 104 weeks at doses of 0, 50, 100 or 200 ppm. Male and female mice in the CMP-exposed groups had decreased body weight but no noticeable clinical signs when compared with the control group. Dose-related increases in the incidences of gastric mucosal hyperplasia and squamous cell papilloma were observed in both sexes, and squamous cell carcinoma was observed in only one male mouse in the 100 ppm group. An increased incidence of Harderian gland adenoma in female mice was also recognized. In the nasal cavity, eosinophilic exudate associated with atrophy of olfactory epithelia, respiratory metaplasia of olfactory epithelia and olfactory gland, and eosinophilic changes in respiratory and olfactory epithelia were increased in both sexes.

Key words: 3-Chloro-2-methylpropene, BDF1 mouse, Inhalation study, Squamous cell papilloma, Forestomach, Nasal cavity, Harderian gland adenoma

Introduction

3-chloro-2-methylpropene (CMP) has been widely used as an intermediate in the production of plastics, pharmaceuticals and other organic chemicals and as a fumigant insecticide for grains, tobacco and soil^{1,2)}. CMP is a colorless and volatile liquid with a pungent odor. It has a specific gravity of 0.92 at 15°C, a boiling point of 71–72°C, a vapor pressure of 101.1 mmHg at 20°C, and a refractive index of 1.4318 at 15°C²⁾. CMP is soluble in chloroform, acetone, alcohol, ether and benzene²⁾.

According to a National Toxicology Program Technical Report (1986), a long-term toxicity study of CMP has been conducted in B6C3F1 mice with administration by the gavage route, and a dose-related increase in gastric (forestomach) inflammation was observed. An increased incidence of epithelial hyperplasia of the forestomach was also observed,

and CMP-induced squamous cell papilloma and squamous cell carcinoma of the forestomach were found in mice. It is highly probable that workers are exposed to CMP while using it as a gaseous insecticide or as an intermediate in organic synthesis. Recently the U.S. Environmental Protection Agency nominated CMP for carcinogenicity testing, because of its presence in ambient air and its structural relationship to vinyl chloride, which is a recognized animal and human carcinogen^{3,4)}.

As mentioned above, CMP is a very volatile liquid, and inhalation is the major route of potential occupational exposure in man. Moreover, the data available concerning long-term inhalation of CMP are extremely limited. In the present study, BDF1 mice were exposed for 104 weeks to CMP by the inhalation route, and the results obtained are reported here.

The present paper is the first report to show the occurrence of CMP-induced nasal lesions in BDF1 mice.

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Materials and Methods

Chemical and administration method used

CMP was obtained as a special quality product (Japan Industrial Standard, purity: 95%) from Wako Pure Chemical Industries, Ltd., Tokyo, Japan. It was received as a liquid in 500 g bottles and was stored at room temperature in a testing laboratory room throughout the present study.

Male and female BDF1 (C57BL/6NCrj $\times$ DBA/2NCrj) mice were obtained from Charles River Japan Inc. (Kanagawa, Japan) at 4 weeks of age. They were acclimated for 2 weeks before the start of the experiment, and exposed to CMP at 6 weeks of age. The mice were housed in barrier system animal room maintained at a temperature of $24 \pm 1^\circ\text{C}$ and a relative humidity of $50 \pm 5\%$ with 15–17 air changes per hour. Fluorescent lighting was controlled automatically to give a 12-hr light/dark cycle. All the mice had free access to a standard commercial diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water.

The mice were maintained in the study for 2 years and then killed, and necropsy examinations were performed on all the mice at 110–111 weeks of age. Necropsy was also performed on all mice found dead and on those that were moribund and sacrificed during the course of the study.

Exposure chambers

Throughout the study period, the mice were housed individually in stainless-steel wire mesh cages (224W $\times$ 212D

$\times$ 120H mm) placed within a 3700-L stainless-steel and glass inhalation chamber. The chamber temperature ($24 \pm 2^\circ\text{C}$), the relative humidity of the chamber air ($55 \pm 10\%$), the air flow rate (740 L/min) and the pressure in the chamber ($-5 \sim -15 \text{ mm H}_2\text{O}$) were measured every day at one hour intervals in the case of each group, and were strictly maintained⁵⁾.

Generation and monitoring of the chamber concentration of CMP

CMP vapor was generated from a reservoir by heating the liquid CMP with a constant temperature circulator, and at the same time, the liquid CMP was bubbled with fresh air. The vapor was stabilized by cooling and then heating again, and the CMP concentration was adjusted by dilution with fresh air through an in-line mixer to obtain the target concentration for each exposure chamber⁵⁾ (Fig. 1). The concentration of CMP in each chamber was monitored by gas chromatography at 15-minute intervals. Deviation from the target concentration in each of the chambers was minimal (Table 1).

Experimental design

Groups of 50 male and 50 female mice were exposed to air containing CMP at a concentration of 50, 100 or 200 ppm, 6 hours per day, 5 days per week for 104 weeks. As a control, groups of 50 mice of both sexes were handled in the same manner and were exposed to clean air in similar chambers.

The mice were observed every day to assess their clinical

Fig. 1. 3-Chloro-2-methylpropene vapor generation and inhalation system

Table 1. Actual concentration of 3-chloro-2-methylpropene in the inhalation chambers

Group Name	Concentration (ppm)
	Mean $\pm$ S.D.
Control group	0.0 $\pm$ 0.0
50 ppm group	49.6 $\pm$ 0.3
100 ppm group	99.5 $\pm$ 0.4
200 ppm group	199.8 $\pm$ 0.8

signs, and their behavioral changes and mortality were checked once a day. They were weighed once a week for the first 13 weeks of the study and every 4 weeks thereafter.

Histopathological studies were thoroughly performed on all organs (skin, nasal cavity, nasopharynx, larynx, trachea, lung, bone marrow, lymph node, thymus, spleen, heart, tongue, salivary gland, esophagus, stomach, small intestine, large intestine, liver, pancreas, kidney, urinary bladder, pituitary, thyroid, adrenal gland, testis, epididymis, seminal vesicle, prostate, ovary, uterus, vagina, mammary gland, brain, spinal cord, peripheral nerve, eye, Harderian gland, muscle and bone) of the experimental animals and they were weighed at the time of scheduled necropsy. All organs and tissues for microscopic examinations were fixed in 10% neutral buffered formalin, and embedded in paraffin by routine procedures. Thin tissue sections were prepared and stained with hematoxylin and eosin and with special stains where appropriate.

Statistical analysis

In body weight and organ weight data, and analysis of continuous variables, the following methods were used. The Bartlett method was used to test whether the variance of the data obtained, comparing the control and treated groups, was statistically different. When the variance was not statistically different, the one-way layout analysis of variance was performed. When the difference was statistically significant, the significance of the difference in the group mean was analyzed by Dunnett's multiple comparison test. When the variance was statistically different, the Kruskal-Wallis rank sum test was performed by arranging all data for the control and treated groups in descending order. When the difference between the groups was statistically significant, Dunnett's multiple comparison test by rank was performed. Two-sided analysis with a P-value of 0.05 was performed in the case of preliminary tests, whereas two-sided analysis with a P-value of 0.05 and 0.01 was performed in the case of final tests.

In histopathological data, non-neoplastic lesions were analyzed by the chi-squared test, by classifying animals

without lesions into grade 0. The chi-squared test was performed in comparing the control with each of the treated groups. Neoplastic lesions were also analyzed by the Peto test, the Cochran-Armitage linear trend test, and Fisher's exact test, on a given kind of tumor found in over 5% of the animals in at least one group⁶⁾.

Results

Survival rate, body weight, clinical signs and food consumption

Survival rates of the male and female mice in all groups exposed to CMP in this study were slightly higher than those of the mice in the control group. That is, at the end of the experiment, the survival rate of male mice was 80.0% (40/50) in the control group, 88.0% (44/50) in the 50 ppm group, 86.0% (43/50) in the 100 ppm group, and 92.0% (46/50) in the 200 ppm group. The survival rate of female mice was 58.0% (29/50) in the control group, 60.4% (29/48) in the 50 ppm group, 62.0% (31/50) in the 100 ppm group, and 67.3% (33/49) in the 200 ppm group.

A relatively significant decrease in body weight was observed in all exposed groups throughout the experiment period, and it was more marked in males than in females (Figs. 2 and 3), but no noteworthy clinical signs were detected in any of the mice in this study.

The amount of food intake in all exposed groups was slightly less than that in the control group throughout the experiment period, and no significant difference according to sex was recognized.

Macroscopic findings on examination of internal organs

An increased incidence of mucosal polypoid lesions in the forestomach was observed in both male and female mice in the 100 ppm and 200 ppm groups. No other noticeable abnormalities were detected macroscopically in any of the animals in this study.

Organ weight

In males, the body weight ratio of the lungs was $0.567 \pm 0.119\%$ in the 100 ppm group and $0.690 \pm 0.717\%$ in the 200 ppm group, and these values were larger than that in the control group ($0.482 \pm 0.128\%$). Nevertheless, a decrease in absolute organ weight was observed in the spleen and liver in all experimental groups, in the heart and kidneys in the 100 ppm and 200 ppm groups, and in the brain in the 200 ppm group. The decrease in the body weight ratio of these organs were not correlated to the exposed concentration of CMP, and, on the other hand, the body weights ratio of the heart and brain were greater than those of the control group.

Fig. 2. Body weight changes of male mice in the 2-year inhalation study of 3-chloro-2-methylpropene

Fig. 3. Body weight changes of female mice in the 2-year inhalation study of 3-chloro-2-methylpropene

In females, the body weight ratio of the lungs was $0.808 \pm 0.160\%$ in the 100 ppm group, and $0.961 \pm 0.508\%$ in the 200 ppm group, which were also greater than that of the control group ($0.725 \pm 0.124\%$). A decrease in absolute organ weight was observed in the heart, kidneys and brain in the 100 ppm and 200 ppm groups, and in the spleen and liver in the 200 ppm group. No remarkable difference between the experimental and control groups was seen in the body weight ratio of these organs, and the body weight ratio of the brain was greatest in the 100 ppm group.

Pathology

Neoplastic lesions of the stomach and the Harderian gland were detected, and non-neoplastic lesions of the nasal cavity and the stomach were observed and their incidences are shown in Tables 2 and 3.

Squamous cell papilloma of the forestomach was observed, appearing as a white polypoid and/or cauliflower-shaped mass on the gastric mucosa macroscopically. Histopathologically, there were noticeable hyperkeratosis with parakeratosis. Some of them had a narrow stalk, and some had a broad base and their stroma contained typical finger-

Table 2. Summary of neoplastic lesions in the 2-year inhalation study of 3-chloro-2 methylpropene (BDF1 mice)

Concentration of CMP	Male				Female			
	Control	50 ppm	100 ppm	200 ppm	Control	50 ppm	100 ppm	200 ppm
No. of animals investigated	50	49	50	50	50	48	50	49
Neoplastic lesions								
Stomach: Squamous cell papilloma	1 (2%)	0	3 (6%)	4 (8%)	1 (2%)	0	5 (10%)	4 (8%)
Squamous cell carcinoma	0	0	1 (2%)	0	0	0	0	0
Harderian gland: adenoma	3 (6%)	7 (14%)	9 (18%)	5 (10%)	0 (0%)	4 (8%)	7 (14%)	8 (16%)

Table 3. Summary of selected non-neoplastic lesions in the nasal cavity and stomach in the 2-year inhalation study of 3-chloro-2-methylpropene (BDF1 mice)

Experimental group Concentration of CMP	Male				Female			
	Control	50 ppm	100 ppm	200 ppm	Control	50 ppm	100 ppm	200 ppm
No. of animals investigated	50	50	50	50	50	48	50	49
Nasal cavity								
Eosinophilic exudate	0	0	6	23	0	5	23	43
Atrophy of olfactory epithelia	0	0	3	21	0	2	9	39
Respiratory metaplasia of olfactory epithelia	9	4	15	49	2	5	15	49
Respiratory metaplasia of olfactory gland	4	3	5	48	4	9	6	49
Intracytoplasmic eosinophilic globules (respiratory epithelia)	7	31	36	47	38	40	40	49
Intracytoplasmic eosinophilic globules (olfactory epithelia)	7	11	23	33	24	19	45	41
Stomach								
Mucosal hyperplasia of forestomach	3	0	9	21	0	1	4	19

Fig. 4. Typical squamous cell papilloma of the forestomach.
200 ppm-CMP exposed female mouse. H&E stain.

like projections (Fig. 4). Squamous cell papilloma was detected more frequently in the mice of both sexes exposed to CMP than in control mice. These findings were confirmed to be statistically significant by the Peto test.

A squamous cell carcinoma of the forestomach was encountered, appearing as a firm white irregular mucosal protrusion with central ulceration, in one male mouse in

Fig. 5. Well differentiated squamous cell carcinoma of the forestomach.
Invasion into the gastric submucosa is recognizable. 100 ppm-CMP exposed male mouse. H&E stain.

the 100 ppm group. Microscopically it had a broad base, and was assumed to be arising from a preexisting papilloma. It was a well-differentiated type of carcinoma and locally invasive (Fig. 5).

Epithelial hyperplasia of the forestomach was observed,

Fig. 6. Diffuse mucosal hyperplasia of the forestomach.

Hyperkeratosis, prominent keratohyaline layer and marked elongation of rete ridges are demonstrated. 200 ppm-CMP exposed female mouse. H&E stain.

Fig. 7. Adenoma of the Harderian gland.

Adenoma shows papillary and expansive growth pattern. 200 ppm-CMP exposed female mouse. H&E stain.

Fig. 8. Exudate in the nasal cavity.

An eosinophilic homogenous exudate occupies the air space, however, no inflammatory cells are detectable. 200 ppm-CMP exposed female mouse. H&E stain.

Fig. 9. A marked atrophy of olfactory epithelia observed in the ethmoturbinate of the nasal cavity.

200 ppm-CMP exposed female mouse. H&E stain.

appearing as a diffuse thickening of all squamous epithelial layers without any cellular or structural atypia and local invasion (Fig. 6). The incidence of this finding was significantly higher only among the mice in the 200 ppm group compared with the control group in both sexes (Table 3). The incidence of epithelial cell hyperplasia of the forestomach in the 100 ppm group was slightly higher than that in the control group in both sexes.

Harderian gland adenoma which had a papillary growth pattern with compression of the surrounding tissue was encountered. The adenoma had slight cellular atypia, and alveolar epithelia were pseudostratified or stratified (Fig. 7). Harderian gland adenoma was detected more frequently in female mice exposed to CMP than in female mice in the

control group (Table 2). The difference was statistically significant as indicated by the Peto test, the Cochran-Armitage test and Fisher's exact test.

Pathological findings in the nasal cavity are summarized in Table 3. The incidence of eosinophilic exudate in the nasal cavity seemed to be increased dose dependently. The eosinophilic exudate was homogenous in nature without inflammatory cells and accumulated in the level 3 lumen usually in the dorsal meatus (Fig. 8).

Atrophy of the olfactory epithelia was observed in some of the mice with eosinophilic exudate, and increased dose-dependently in the treated groups (Fig. 9). This finding was found frequently in the anteriodorsal portion of the olfactory mucosa in the dorsal meatus. Respiratory epithelial metaplasia of the olfactory epithelia (Fig. 10) and of the

Fig. 10. Respiratory metaplasia of olfactory epithelia (arrows) observed in the dorsal wall of level 3 of the nasal cavity. 200 ppm-CMP exposed female mouse. H&E stain.

Fig. 11. Respiratory metaplasia of olfactory gland (arrows) observed in the dorsal wall and dorsal septum of level 2 of the nasal cavity. 200 ppm-CMP exposed female mouse. H&E stain.

Fig. 12. (a): Normal respiratory ciliated epithelia, control mouse. (b): The cytoplasm of respiratory epithelia are filled with eosinophilic materials. 200 ppm-CMP exposed female mouse. H&E stain.

Fig. 13. (a): Normal olfactory epithelia, control mouse. (b): The cytoplasm of olfactory epithelia are filled with eosinophilic materials. 200 ppm-CMP exposed female mouse. H&E stain.

olfactory gland (Fig. 11) were frequently detected in the 200 ppm group in both sexes. Some intracytoplasmic eosinophilic globules were found in respiratory and olfactory epithelial cells (olfactory sustentacular cells) even in the control mice. The number and size of the globules were conspicuously increased in mice with chronic exposure to CMP (Figs. 12, 13). These globules were brightly eosinophilic in hue on hematoxylin and eosin staining, showing weak positive staining in the case of both PAS and diastase-PAS stains, no staining in the case of alcian blue or mucicarmine stains, weak orange-yellow staining in the case of Masson's trichrome stain, scarcely positive staining in the case of Congo red stain, and faint blue staining in the case of toluidine blue stain (pH 4.1). They appeared dark blue in respiratory epithelia and light blue in olfactory epithelia on staining with toluidine

blue stain (pH 7.0). These globules showed no sign of staining in the case of PTAH stains (Table 4).

There were no apparent neoplastic or non-neoplastic lesions in the other organs investigated.

Discussion

According to a National Toxicology Program Technical report (NTP)³, NTP carried out toxicology and carcinogenicity studies of CMP in B6C3F1 mice. In those studies, groups of 50 male and 50 female mice were administered CMP in corn oil by gavage at doses of 0, 100, and 200 mg/kg body weight, 5 days per week for 104 weeks. The mean body weights of male mice administered the high dose and of female mice in both CMP-treated groups were slightly lower (5%–9%) than those of the controls, whereas the survival rate of both

Table 4. Histological and histochemical findings of intracytoplasmic eosinophilic globules in respiratory and olfactory epithelia in the nasal cavity (BDF1 mice)

	Respiratory epithelia	Olfactory epithelia
Hematoxylin and eosin	brightly eosinophilic	brightly eosinophilic
Periodic acid-Schiff	weak positive	weak positive
PAS after diastase digestion	weak positive	weak positive
Alcian blue (pH 2.5)	negative	negative
Mucicarmine	negative	negative
PTAH	negative	negative
Masson's trichrome	orange yellow	orange yellow
Congo red	scarcely positive	scarcely positive
toluidine blue (pH 2.5)	negative	negative
toluidine blue (pH 4.1)	faintly blue	faintly blue
toluidine blue (pH 7.0)	dark blue	light blue

male and female mice was not affected by CMP administration. A dose-related increase in the incidence of forestomach inflammation was observed in mice of both sexes. An increased incidence of forestomach basal cell hyperplasia was observed in mice of each sex. CMP-induced squamous cell papillomas and squamous cell carcinomas of the forestomach were noticed in mice administered the high dose. Invasion and/or metastasis of the squamous cell carcinomas to other organs were observed^{7,8)}. The incidences of inflammation of the nasal cavity and of nephropathy and/or nephrosis were greater in the two CMP-treated groups than in the control groups of mice of both sexes³⁾.

In view of the NTP data, we have performed chronic toxicity and carcinogenicity studies of CMP in BDF1 mice by the inhalation route. Remarkably, the changes observed in the target organs were almost identical to those described in the NTP report, even though the route of administration of CMP in our study was different from that in the NTP study. Male and female mice in the CMP-exposed groups showed decreased body weight without any noticeable clinical signs when compared with the control group. Noteworthy pathological changes were recognized in the forestomach and nasal cavity.

In the present study, the incidence of forestomach tumors in mice of both sexes was significantly higher in the high-dose groups. The preneoplastic and neoplastic lesions encountered were epithelial cell hyperplasia and squamous cell papilloma and these were observed in mice in the 100 and 200 ppm groups. The incidence of squamous cell papilloma was higher than that in the case of the Japan Bioassay Research Center's historical control data for BDF1 male and female mice (male: 0.0%, female: 2.0%). Only one case of squamous cell carcinoma was observed in a male mouse in the 100 ppm group. It is conceivable that these

changes were induced by CMP inhalation. It has been suggested that epithelial hyperplasia of the forestomach may progress to become a papilloma, and subsequently a carcinoma, in many previous reports^{3, 9-16)}. In the present study, it was similarly suggested that the neoplastic lesions of the forestomach progressed with such a pattern. The gastric lesions that developed in the present CMP inhalation study were similar to those observed in the study performed by the gavage route, but the severity was milder than that in the case of the gavage route. It is known that an inhaled chemical substance deposits in the upper respiratory tract and some of it is transported to the digestive tract¹⁷⁾. Also in the present inhalation study, some of the CMP deposited in the nasal cavity may have been transported to the stomach and have induced the stomach lesions.

The incidence of Harderian gland adenoma in female mice was significantly high in the 100 and 200 ppm groups in the present study. The incidence of such Harderian gland tumors was slightly higher than the maximum incidence (12.0%) of this tumor in the historical control data of the Japan Bioassay Research Center for BDF1 female mice. CMP may therefore be an etiologic agent of this tumor. Because Harderian gland adenoma was not detected in the NTP studies of CMP, the frequent occurrence of this tumor in the present study may be attributable to inhalation of CMP.

With respect to the nasal lesions, increase in incidences of eosinophilic exudate, atrophy of olfactory epithelia, respiratory metaplasia of olfactory epithelia and the olfactory gland, and intracytoplasmic eosinophilic globules were indicated. Eosinophilic exudate in the nasal cavity was limited to the posterior part of the cavity. Eosinophilic exudate was considered to be one of the specific changes observed in the present CMP inhalation study. Eosinophilic exudate associated with atrophy of the olfactory epithelia may have occurred as

a result of degeneration of the olfactory epithelial cells. Respiratory metaplasia of the olfactory epithelia and of the olfactory gland were observed at 200 ppm in both sexes. It was reported that olfactory epithelia were found to be replaced by respiratory-like mucosal epithelia which possibly means that olfactory epithelia that had lost their sensory cells¹⁸). The respiratory metaplasia of olfactory epithelia and of the olfactory gland have been reported for rats as an aging change and an adaptive response^{19, 20}). An increase in the abundance of intracytoplasmic eosinophilic globules in the respiratory and olfactory epithelia was also observed at significantly high incidences in the groups exposed to CMP. The exact nature and chemical composition of this eosinophilic material has not yet been determined, but it is conceivable that these globules represent a proteinaceous material secreted and accumulated in these cells¹⁹⁻³⁷). These findings might be closely related to the administration of CMP by the inhalation route. This change was thought to be an adaptive response to CMP inhalation and its occurrence increased with age.

An increase in the body weight ratio of the lungs was observed in mice in all experimental groups exposed to more than 100 ppm of CMP. But no histopathological pulmonary findings correlated to the organ weight change were obtained. Histopathological changes in the nasal cavity, including a large amount of eosinophilic exudate were detected, and the changes seemed to be due to the increased lung weight. Concerning the other organs, a decrease in absolute organ weight was recognized in the spleen, liver, heart, kidneys and brain in males, and in the heart, kidneys, brain, spleen and liver in females. In the present experimental study, an increase in body weight ratio was not recognized in the mentioned above organs, and the groups with decreased absolute weight of these organs had lower body weight than those in the control group. It is therefore conceivable that the decrease in organ weight in these groups was closely related to the decreased body weight of the mice.

We have conducted a 2-year CMP-inhalation study in mice, and found an increased incidence of mucosal hyperplasia of the forestomach in the group of both sexes exposed to 200 ppm, an increased incidence of squamous cell papilloma of the forestomach in the groups of both sexes exposed to 100 or 200 ppm, and one case of squamous carcinoma in a male mouse exposed to 100 ppm. Other noteworthy neoplastic findings were: Harderian gland adenomas in female mice were significantly increased in the exposed groups. In the nasal cavity, eosinophilic exudate, atrophy of olfactory epithelia, respiratory metaplasia of olfactory epithelia and the olfactory gland and, intracytoplasmic eosinophilic globules in respiratory and olfactory epithelial cells were increased by CMP.

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