Further Carcinogenicity Study on Methylmercury Chloride in ICR Mice

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(Received 20 September 1985/Accepted 12 November 1985)

ABSTRACT. Further carcinogenicity study on methylmercury chloride (MMC) was conducted in ICR mice (60/sex/group) to confirm our earlier finding that MMC was a renal carcinogen in male ICR mice. MMC was fed to mice at dietary levels of 0, 0.4, 2, or 10 ppm for 104 weeks. Renal tumors, mostly adenocarcinomas, were induced only in male mice of the 10 ppm group. The first case with a renal tumor was observed at 58 weeks of the study. The tumor incidence was 50% (13/26) in the 10 ppm group. Average intake of MMC in this group was 0.906 mg/kg/day. No renal tumors were induced in other groups including females.—**KEY WORDS:** carcinogenicity, ICR mouse, methylmercury chloride, renal carcinogen, sex difference.

Jpn. J. Vet. Sci. 48(1): 127–135, 1986

Methylmercury chloride (MMC) is one of the major causative agents of Minamata Disease. We have conducted long-term feeding studies in rats and mice to examine comparative aspects of cumulative toxicity as well as neurotoxicity of MMC [8–10]. During the course of our studies we unexpectedly obtained a result suggesting renal carcinogenic potentiality of MMC in male mice fed diet containing 15 ppm of this compound [8].

We conducted a further study using lower dose levels of MMC to confirm our finding in the previous study [8].

MATERIALS AND METHODS

Methylmercury chloride (MMC), 99.3% in purity, was supplied from Tokyo Kasei Kogyo Co., Ltd., Japan. Male and female SPF ICR mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Japan, at 4 weeks of age. Four animals of either sex were housed in an aluminum cage with wire-mesh floor in a barrier system animal room (controlled temperature at $24 \pm 1^\circ\text{C}$ and humidity at $55 \pm 5\%$). After one-week acclimatization, 60 males and 60 females were allotted to each dose group.

MMC was mixed with a commercial powdered chow M (Oriental Yeast Ltd., Japan) at 0 (control), 0.4, 2, or 10 ppm. MMC was fed to mice for 104 weeks. During the study, 6 males and 6 females from each group were killed by design at intervals of 26 weeks to be subjected to histopathological examination. All surviving animals at termination of the treatment were subjected to the same examination.

All animals were observed daily for their general conditions. Animals found dead and killed *in extremis* were autopsied immediately. Body weight of each animal was recorded weekly during the first 26 weeks of the treatment and biweekly thereafter. Food consumption was measured twice a week for designated cages (8/sex/group).

Organs and tissues listed in Pesticide Assessment Guidelines of U.S. Environment Protection Agency [16] were fixed in 10% formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin for microscopic examination. Histopathology was conducted on all animals found dead, killed *in extremis*, or killed by design. Fisher's exact probability test or chi-square test with Yates' correction was

used for determining the significance of the results between the concurrent control group and each MMC-treated group.

RESULTS

No animals showed neurotoxic signs. Although male mice in the 10 ppm group died by 98 weeks, there were no marked differences in mortality between treated groups and the control group (Fig. 1). The body weight and food consumption in both sexes in the treated groups were comparable to those of the controls throughout the study (Fig. 2). The overall mean daily intakes of MMC (mg/kg) in the 10, 2 and 0.4 ppm groups were 0.906, 0.188 and 0.0375 in males, and 0.785, 0.144 and 0.0318 in females, respectively.

An increase in the number of animals showing granular surface, swelling and discoloration of the kidney was observed in males of the 10 ppm group after 26 weeks. A renal tumor mass was first encountered in a male of the 10 ppm group killed *in extremis* at

58 weeks. Among 26 males in this group which died or were killed after 58 weeks, 6 animals had renal masses. No renal masses were macroscopically found in females of this group and either sex of other groups including the control group. Three of the 6 males had white, solid and well demarcated unilateral masses less than 1 cm in diameter in the renal cortex. The rest disclosed large multilobular cystic masses which were composed of white small nodules and cysts of various sizes filled with blackish brown fluid. Except for these lesions and neoplastic masses in the kidney, there were no significant increases in the incidence of macroscopic findings in the treated groups as compared to the control.

Toxic epithelial degeneration of the renal proximal tubules characterized by nuclear swelling and vacuolated cytoplasm was histologically seen in both sexes of the 10 ppm group which were subjected to interim kill at 26 weeks. The severity of this degenerative change became more marked with prolongation of the treatment. Regenerative changes

Fig. 1. Survival curves in mice fed diets containing MMC for 104 weeks. Survival rate is expressed as ratios of survivors to used animals with exception of ones for interim kill.

Fig. 2. Group mean body weights in mice fed diets containing MMC for 104 weeks.

of the proximal tubular epithelium consisting of vesicular nuclei and basophilic cytoplasm were concurrent with these degenerative changes. Both lesions were more prominent in males than in females. The overall incidence of these nephrotoxic changes was 40 of 59 males and 19 of 60 females in the 10 ppm group. Similar but mild nephrotoxic changes were also observed in males of the 2 ppm group. The incidence was 12 of 58 males, which was lower than that in the 10 ppm group.

Focal hyperplasia of tubular epithelium (Fig. 3), which was composed of some tubules filled with epithelial cells or dilated tubules lined by columnar cells with large eosinophilic or glassy cytoplasm and a few atypical nuclei, was observed in males of the 10 ppm

group after 47 weeks. Cystic kidney was also seen after 62 weeks. The incidences of these changes were 13/59 and 8/59, respectively, while no animals showed the changes in the control group. Urinary cast and pelvic dilatation in the kidney were seen with increased incidences in males of the 10 ppm and/or 2 ppm groups.

In the 10 ppm group, the incidences of decreased spermatogenesis in males and degeneration or fibrosis of the sciatic nerve in females were 11/59 and 23/60, respectively, and increased significantly as compared to the control group (1/58 and 9/19, respectively).

Besides the above lesions, various kinds of spontaneous lesions were observed in animals of both treated and control groups. However, the incidences of spontaneous lesions were

Fig. 3. Focal hyperplasia of the renal tubular epithelium. Male mouse in the 10 ppm group killed *in extremis* at 47 weeks. H-E stain. $\times 300$.

Fig. 4. Solid type of adenocarcinoma with the pseudoglandular pattern surrounded by delicate stroma. Cellular and nuclear pleomorphism is a prominent feature in the neoplastic cells. Male mouse in the 10 ppm group killed *in extremis* at 92 weeks. H-E stain. $\times 150$.

comparable to the controls.

All tumors observed histologically in this study were shown in Table 1.

Renal epithelial tumors were seen in males of the 10 ppm group which died or were killed after 58 weeks. The most renal masses observed macroscopically in this group were classified as solid type or cystic papillary type of adenocarcinomas. In solid type, pleomorphism of cellular and nuclear structure was prominent in tumor cells and mitotic figures were sometimes observed (Fig. 4). There were occasional areas showing a cord-like or trabecular pattern resembling the renal tubules. No invading proliferation of the tumor cells was noticed in the surrounding tissues. Cystic papillary type of adenocarcinoma was composed of polyhedral, columnar or cuboidal cells with bizarre nuclei and glassy or vacuolated cytoplasm (Fig. 5). Severe hemorrhagic or necrotic foci were frequently ob-

served in these tumors (Fig. 6). Especially in the large cysts where the tumor cells showed an extensive papillary growth, hemorrhagic changes were severe. In addition, these types of adenocarcinomas were also seen in males of the 10 ppm group in which tumor masses could not be detected at autopsy. However, metastasis of renal adenocarcinomas to the lung, liver and regional lymph nodes was not observed. The total number of male animals with renal adenocarcinomas was 10/59 in the 10 ppm group. Additionally, 3 males in this group had renal adenomas which were composed of columnar cells disclosing tubular arrangement (Fig. 7). Among these 13/59 animals with renal tumors, 2 of the males had bilateral adenocarcinomas. There were no renal tumor bearers in males of other treated groups, although one male in the control

Table 1. Incidence of tumors in mice fed diets containing MMC for 104 weeks

Dose level (ppm) Number of mice examined	Number of mice with tumors							
	Male				Female			
	0 58	0.4 59	2 58	10 59	0 59	0.4 60	2 60	10 60
Blood and hematopoietic system								
Spleen: Hemangioma	0	0	0	0	1	0	2	0
Hemangiosarcoma	1	1	0	0	1	0	0	0
Lymphnode: Malignant lymphoma	0	0	0	0	0	1	0	0
Myelogenous leukemia	1	0	0	0	1	1	1	0
Malignant lymphoma	1	3	3	2	8	11	8	9
Respiratory system								
Lung: Adenoma	7	1 ^{a)}	2	0 ^{b)}	1	2	2	1
Adenocarcinoma	8	4	8	6	5	4	8	12
Digestive system								
Liver: Hepatocellular adenoma	17	15	14	5 ^{b)}	17	18	16	8 ^{a)}
Hepatoblastoma	0	0	1	0	0	0	0	0
Hemangioma	0	0	1	0	0	1	0	0
Hepatocellular carcinoma	1	1	2	4	1	3	2	2
Fibrosarcoma	0	0	1	0	0	0	0	0
Malignant lymphoma	0	0	0	0	1	0	0	0
Stomach: Papilloma	1	0	0	0	0	0	0	0
Intestine: Adenoma	0	0	0	0	0	0	0	1
Hemangioma	0	0	0	0	1	0	0	0
Uro-genital system								
Kidney: Adenoma	1	0	0	3	0	0	0	0
Adenocarcinoma	0	0	0	10 ^{c)}	0	0	0	0
Urinary bladder: Transitional cell carcinoma	0	0	0	1	0	0	0	0
Ovary: Adenoma	—	—	—	—	2	1	0	1
Granulosa cell tumor	—	—	—	—	1	1	0	0
Fibroma	—	—	—	—	1	0	0	0
Uterus: Polyp	—	—	—	—	2	1	2	4
Stromal cell tumor	—	—	—	—	1	0	0	0
Leiomyoma	—	—	—	—	0	1	0	0
Hemangioma	—	—	—	—	0	0	0	1
Leiomyosarcoma	—	—	—	—	1	0	0	0
Endocrine system								
Pituitary: Anterior adenoma	0	0	0	0	0	1	0	0
Thyroid: Adenoma	1	0	0	0	0	0	0	0
Adrenal: Cortical adenoma	0	0	0	1	0	0	0	0
Nervous system								
Brain: Meningioma	0	0	0	0	0	1	0	0

a, b, c) Significantly different from the control group at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Table 1. (continued)

Dose level (ppm) Number of mice examined	Number of mice with tumors							
	Male				Female			
	0 58	0.4 59	2 58	10 59	0 59	0.4 60	2 60	10 60
Skin/Subcutis								
Papilloma	0	2	1	0	0	0	0	0
Acanthoma	0	0	0	0	0	1	0	0
Mammary adenoma	1	0	0	0	0	0	0	1
Hemangioma	0	0	0	1	0	0	0	0
Mammary adenocarcinoma	0	0	0	0	4	0	2	1
Fibrosarcoma	0	0	0	1	1	0	0	0
Rhabdomyosarcoma	1	1	0	0	1	2	1	0
Hemangiosarcoma	0	0	0	0	0	1	0	0
Others								
Harderian gland: Adenoma	3	0	2	1	2	3	2	2
Bone: Osteoma	0	0	0	0	0	0	0	1
Benign tumor bearers	22	16	18	11 ^{a)}	22	22	20	18
Malignant tumor bearers	12	9	11	19	20	21	20	20
Total tumor bearers	27	21	24	27	32	34	33	31

a) Significantly different from the control group at $p < 0.05$.

Fig. 5. Cystic papillary type of adenocarcinoma, showing papillary proliferation and cellular pleomorphism. Male mouse in the 10 ppm group subjected to interim kill at 78 weeks. H-E stain. $\times 150$.

group had a unilateral renal adenoma. No females in the treated and control groups developed any renal tumors. The overall incidence of these tumor bearers *versus* effective number of mice after 58 weeks was 50% (13/26) in the 10 ppm group, and was exceedingly higher than the control group (Table 2).

In addition to renal tumors, various kinds of tumors were detected in the treated and control groups. However, the incidences of these tumors in the treated groups showed no statistically significant increases as compared to the control group (Table 1).

DISCUSSION

The results of the present studies confirmed those of our previous study in which renal carcinogenicity of MMC was found in male mice [8]. It seems noteworthy that MMC at a very low level of 10 ppm (0.906 mg/kg/day) could induce renal cancers in male mice.

It has been reported that organomercury

Fig. 6. Cystic papillary type of adenocarcinoma, showing marked papillary proliferation and hemorrhagic or necrotic foci. Male mouse in the 10 ppm group killed *in extremis* at 76 weeks. H-E stain. $\times 28$.

Fig. 7. Renal adenoma with tubular pattern. Male mouse in the 10 ppm group subjected to interim kill at 78 weeks. H-E stain. $\times 150$.

compounds produced chromosome or DNA damage in mammalian cells (7, 12, 14, 17). In addition, positive results of methylmercury were obtained in the rec-assay with *Bacillus subtilis* (6) and sister-chromatid exchange analysis (11). However, methylmercury might not induce gene mutation since negative results were obtained in the Ames test using *Salmonella typhimurium* (6). Therefore, the mechanism of renal carcinogenesis in mice remains to be obscure.

Several long-term feeding studies with a small amount of methylmercury have been conducted in the monkey [5], cat [2] and rat [13, 18]. However, no increased incidences of any tumors were reported in these studies. We have also conducted a life span feeding study with MMC in rats at dietary levels of 0, 0.4, 2, or 10 ppm for 30 months [9, 10]. However, there were no increases in the incidence of renal tumors in the treated groups of either sex, although the kidney has been recognized to be one of the target organs

Table 2. Incidence of renal tumors in male mice

	Dose level (ppm)			
	0	0.4	2	10
Adenoma	1 ^a /32 ^b (3) ^c	0/25(0)	0/29(0)	3/26 (12)
Adenocarcinoma	0 /32 (0)	0/25(0)	0/29(0)	10/26 ^d (38)
Renal epithelial tumor	1 /32 (3)	0/25(0)	0/29(0)	13/26 ^d (50)

a) Number of mice with renal tumor.

b) Effective number of mice (No. of animals killed or found dead after 58 weeks).

c) Percentage.

d) Significantly different from the control group at $P < 0.001$.

of organomercury compounds in rats [3] and marked renal toxic injuries were seen in animals of the 10 ppm group [10]. These results may suggest that there is a species difference in sensitivity to oncogenic potentiality of methylmercury between mice and rats.

Focal hyperplasia of the renal tubular epithelium observed in males of the 10 ppm group at a slightly higher rate was considered to be a reparative change following the nephrotoxic change, since they could not be found at the early stage of the degenerative change of the tubular epithelium. Some hyperplastic changes of the renal tubules were reported to be a precancerous condition [1, 4]. The morphology of the hyperplastic tubular cells (Fig. 3) observed was clearly different from the regenerative cells and was somewhat similar to that in the renal tumors induced. This evidence in our study may suggest that the renal tumors originate from the focal hyperplasia of the tubular epithelium.

We also noted a clear sex difference in the renal tumors induced by MMC. The tubular epithelial damage caused by this compound was more prominent in males than in females. Thomas *et al.* [15] reported that elimination of mercury after a single injection of labeled methylmercury to the rat was faster in females than in males. These findings indicated that males were more prone to cumulative renal injuries of methylmercury compared

with females. Further studies are now in progress to clarify the role of testosterone for the renal carcinogenicity in mice.

ACKNOWLEDGEMENTS. We are greatly indebted to Dr. LeRoy B. Hall, Jr., National Institute of Environmental Health Sciences, U.S.A., for his critical reading of this manuscript.

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要 約

塩化メチル水銀の ICR 系マウスにおける発癌性確認試験: 平野雅裕・三森国敏・真板敬三・白須泰彦 (残留農薬研究所) ——塩化メチル水銀 (MMC) の ICR 系雄マウスに対する腎腫瘍誘発性を確認するため, MMC を 0, 0.4, 2, 10 ppm の濃度で飼料に混入し, 雌雄の ICR 系マウス (各群各性60匹) に104週間投与した。10 ppm 群の雄で, 投与58週以後腎腫瘍 (ほとんどが腺癌) が誘発された。58週以上生存した有効動物に対する担腎腫瘍動物の頻度は, 10 ppm (0.906 mg MMC/kg/日) 群で 50% (13/26例) であった。雄の他の群および雌では腎腫瘍は誘発されなかった。