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Assessment of methyl methanesulfonate using the repeated-dose liver micronucleus assay in young adult rats

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ABSTRACT

A repeated-dose liver micronucleus assay using young adult rats was conducted with methyl methanesulfonate (MMS) as a part of a collaborative study supported by the Collaborative Study Group for the Micronucleus Test/the Japanese Environmental Mutagen Society–Mammalian Mutagenicity Study Group. MMS is a classical DNA-reactive carcinogen, but it is not a liver carcinogen.

In the first experiment (14-day study), MMS was administered per os to 6-week-old male Crl:CD (SD) rats every day for 14 days at a dose of 12.5, 25, or 50 mg/kg/day. In the second experiment (28-day study), 6-week-old male SD rats were treated with MMS at 7.5, 15, or 30 mg/kg/day for 28 days, because the highest dose used in the 14-day study (50 mg/kg/day) caused mortality. Hepatocyte and bone marrow cell specimens were prepared on the day after the final dose. The frequency of micronucleated hepatocytes (MNHEPs) in the liver and that of micronucleated immature erythrocytes (MNIMEs) in the bone marrow were evaluated. Exposure to 50 mg/kg/day MMS for 14 days resulted in an increased frequency of MNHEPs, but MMS had no effect on the frequency of MNHEPs in the rats exposed to the chemical for 28 days at doses up to 30 mg/kg/day. MMS induced MNIMEs production at doses of 25 and 50 mg/kg/day in the 14-day study and at doses of 15 and 30 mg/kg/day in the 28-day study. Overall, the effect of MMS on the frequency of MNHEPs was considered to be equivocal.

1. Introduction

A collaborative study by the Mammalian Mutagenicity Study Group, which is a subgroup of the Japanese Environmental Mutagen Society, was conducted to evaluate the suitability of the repeated-dose liver and gastrointestinal tract micronucleus assays using young adult rats. As a part of that collaborative study, we assessed the effects of methyl methanesulfonate (MMS) using the repeated-dose liver micronucleus assay.

MMS is considered to be a typical DNA-reactive carcinogen. In rats, it was reported that the nervous system is the target tissue for MMS carcinogenicity [1], whereas the liver is not affected. However, there are conflicting results about the genotoxic effects of MMS in the rodent liver. In the mouse liver, MMS produced positive results in the unscheduled DNA synthesis (UDS) test [2] and the comet assay [3,4], but did not induce mutations in the transgenic mouse mutation assay [2–5]. MMS produced negative results in the liver micronucleus test in young rats after a single i.p. injection at doses of up to 80 mg/kg [6]; however, it produced positive results in the liver micronucleus test in partially hepatectomized rats after a single i.p. injection at a dose of 80 mg/kg [7,8].

In our study, the *in vivo* genotoxicity of MMS was evaluated using the repeated-dose liver micronucleus test in young adult rats. The bone micronucleus assay was performed concurrently to evaluate another tissue in the same animals.

2. Materials and methods

2.1. Animals

Four-week-old male Crl:CD (SD) rats were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan) and were used in the experiments at 6 weeks of age. The animals were housed in an air-conditioned room with a 12 h light/dark cycle and 50 ± 20% humidity at 23 ± 3 °C. They were allowed free access to a commercial pellet diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan), and tap water. The study was approved by the Institutional Animal Care and Use Committee of the test facility.

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Table 1
Body weight of rats repeatedly exposed to MMS for 14 days and 28 days.

Duration	Dose (mg/kg/day)	No. of animals	Body weight (g, mean ± SD)				
			Day 1	Day 8	Day 15	Day 22	Day 29
14 days	0	5	198.0 ± 6.5	250.4 ± 5.8	277.8 ± 8.0	–	–
	12.5	5	194.6 ± 17.4	249.0 ± 22.9	278.4 ± 23.3	–	–
	25	5	197.2 ± 7.3	253.0 ± 12.3	280.2 ± 16.8	–	–
	50	5	197.4 ± 12.2	228.0 ± 8.9	247.2 ± 13.0*	–	–
28 days	0	6	188.8 ± 5.9	242.5 ± 11.6	299.5 ± 16.8	341.7 ± 21.0	350.2 ± 24.1
	7.5	6	188.0 ± 7.7	243.5 ± 12.6	295.0 ± 18.3	331.7 ± 24.7	339.8 ± 27.7
	15	6	186.7 ± 6.6	235.3 ± 13.3	285.8 ± 21.8	320.7 ± 25.0	321.0 ± 27.1
	30	6	187.8 ± 8.9	233.7 ± 12.7	289.5 ± 11.0	322.5 ± 13.2	313.5 ± 13.5*

* Significant decrease by Dunnett's test ($p < 0.05$).

2.2. Chemicals

Methyl methanesulfonate (MMS, CAS No. 66-27-3, 99.9% purity) was purchased from Sigma–Aldrich Corporation (MO, USA) and dissolved in physiological saline (Otsuka Pharmaceuticals Factory, Inc., Tokushima, Japan) before use.

2.3. Dose levels and treatment schedule

A 14-day study was conducted at first. The highest dose used for the 14-day study was set at 50 mg/kg/day based on the findings of a previous study, in which the maximum tolerable dose (MTD) of MMS for a 5-day oral dosing protocol involving SD rats was found to be 78 mg/kg/day [9]. In the 14-day study, five animals per group were administered MMS once a day by oral gavage (12.5, 25, or 50 mg/kg/day) or physiological saline for 14 consecutive days. The highest dose for the 28-day study was set at 30 mg/kg/day because the 50 mg/kg/day dose caused mortality in the 14-day study. In the 28-day study, 6 animals per group were administered MMS (7.5, 15, or 30 mg/kg/day) or physiological saline for 28 consecutive days. All dose volumes were set at 5 mL/kg/day.

2.4. Liver micronucleus evaluation

In each study, the rats were euthanized under isoflurane anesthesia at 24 h after the last MMS administration. Hepatocyte preparations were produced using the method reported in the summary paper of this collaborative study [10]. Briefly, the left lateral lobe of the liver was sliced into thin sections and washed with cold Hanks' balanced salt solution (HBSS). Next, the tissue was treated with HBSS containing 100 units/mL of collagenase (Collagenase Yakult-S, Yakult Pharmaceutical Industry, Co., Ltd., Tokyo, Japan) at 37 °C for 1 h to isolate HEPs. Next, the sample was filtered through a gauze and nylon cell strainer (Becton Dickinson and Company, Franklin Lakes, NJ, USA) to remove tissue pieces. The obtained hepatocyte suspension was fixed with 10% neutral buffered formalin.

Immediately prior to microscopic observation, each hepatocyte suspension was mixed and stained with a staining solution consisting of acridine orange (AO, 500 μg/mL) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 10 μg/mL). The resultant mixture was dropped onto a clean glass slide and covered with a coverslip. The specimens were observed under a fluorescent microscope at 400× or 600× magnification with a UV-1A filter (Ultraviolet rays excitation, Nikon Corporation, Tokyo, Japan). The number of micronucleated hepatocytes (MNHEPs) per 2000 parenchymal hepatocytes (HEPs) was counted for each animal. In addition, the number of mitotic phase (M phase) cells per the 2000 HEPs was counted to determine the mitotic index (MI).

2.5. Bone marrow micronucleus evaluation

After the liver had been removed, the femur was extracted from the same animal. Bone marrow cells were collected from the femur according to the method reported by Kawabata et al. [11] with minor modifications. The bone marrow cells were collected by washing the cavity of the femur with ice-cold phosphate buffered saline. The resultant bone marrow cell suspension was filtered through a nylon cell strainer (Becton Dickinson and Company, Franklin Lakes, NJ, USA) to remove any tissue fragments, before being mixed with 10% neutral buffered formalin and stored at room temperature until analysis. The bone marrow cell specimens were examined by researchers who were blinded to the treatment conditions.

Immediately prior to microscopic observation, each bone marrow cell suspension was mixed and stained with an equal volume of AO solution (500 μg/mL). The resultant mixture was dropped onto a clean glass slide and covered with a coverslip. The specimens were observed under a fluorescent microscope at 600× magnification with a B-2A filter (Blue light excitation, Nikon Corporation, Tokyo, Japan). The number of micronucleated immature erythrocytes (MNIMEs) per 2000 erythrocytes was counted for each animal. As a parameter of hematopoietic function in the bone marrow, the proportion of immature erythrocytes (IMEs) among 500 erythrocytes was also calculated for each animal.

2.6. Histopathological examination

The residual liver tissue of the left lateral lobe was fixed with 10% neutral buffered formalin, embedded in paraffin, and then stained with hematoxylin and eosin (H.E.) according to the standard method. The histopathological examination was performed under a light microscope.

2.7. Statistical analysis

Differences in the frequency of MNHEPs and MNIMEs between the experimental and control groups were analyzed using the conditional binomial test reported by Kastenbaum and Bowman [12] at significance levels of 5% and 1%.

The proportion of IMEs among total erythrocytes, MI and body weight at each time point were statistically analyzed with Dunnett's multiple comparison test.

3. Results and discussion

In the group exposed to 50 mg/kg/day MMS for 14 days, one of the five animals showed decreased locomotor activity from Day 14 and died after the body weight measurement on the day of specimen preparation, indicating that the highest dose used for the 14-day study slightly exceeded the MTD. As shown in Table 1, the body weights of the rats exposed to the highest dose of MMS were significantly lower than those of the vehicle control group at the end of the 14-day and 28-day studies. The histopathological liver examinations did not detect any abnormal test substance-related findings in the animals exposed to MMS for 14 or 28 days (Table 2). It was previously reported that no abnormal findings were found in the liver after 28 days repeated exposure to up to 30 mg/kg/day of MMS [13].

The detailed results of the micronucleus evaluation of the liver are shown in Table 3. The mean MI values for the hepatocytes in the vehicle-treated control group were 0.10% and 0.07% for the 14-day and 28-day studies, respectively. The MI values of the MMS-treated groups were similar to those of the vehicle-treated control groups in both studies.

The mean MNHEPs frequencies of the vehicle control group were 0.00 ± 0.00% (mean ± SD) and 0.04 ± 0.08% for the 14-day and 28-day studies, respectively. No MNHEPs were detected in any of the animals in the vehicle control group in the 14-day study, which

Table 2
Histopathological findings in the liver of rats exposed to MMS for 14 days (A) and 28 days (B).

(A)					
Finding	Dose (mg/kg/day)	0	12.5	25	50
	No. of animals	5	5	5	4
No abnormality		5	5	5	4

(B)					
Finding	Dose (mg/kg/day)	0	7.5	15	30
	No. of animals	6	6	6	6
No abnormality		5	6	6	6
Necrosis, focal		1	0	0	0