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Micronucleus induction in rat liver and bone marrow by acute vs. repeat doses of the genotoxic hepatocarcinogen monocrotaline

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ABSTRACT

The liver micronucleus (MN) assay is useful for predicting genotoxic rodent hepatocarcinogenicity. We have recently established the repeated-dose liver MN (RDLMN) assay in rats for integration into general toxicity studies. To investigate the effectiveness of the RDLMN assay, the genotoxic rodent hepatocarcinogen, monocrotaline (MCT), was administered by oral gavage to 6-week old male rats once daily for 14 days at 0.5 and 1.5 mg/kg/day, and for 28 days at 0.15, 0.5, 1.5, 3.75, 7.5 and 15 mg/kg/day. Then, MN induction was measured in the liver and bone marrow (BM), and histopathological hepatotoxicity was examined. Additionally, in order to evaluate the effects of repeated dosing periods on MN inducibility, a double-dose examination of MCT at doses of 15, 30 and 60 mg/kg/day in juvenile (26-days old) and young adult (7-weeks old) rats was also conducted, as an acute dose MN assay. The peripheral blood (PB) and liver were sampled at 48 h and 4 days after the second dosing, respectively. In the repeated-dose MN assay, MCT produced a positive result in the liver at a non-hepatotoxic lower dose level, but not in the BM at any dose level. In contrast, in the double-dose MN assay, MCT showed a negative result in the young adult rat livers, although it gave positive responses in the livers of juvenile rats and in the PB with both age groups. The maximum dose used in the repeated-dose assay was considerably lower than that used in the acute dose assay. These results suggest that a repeated dosing regimen is more suitable for the liver MN assay using young adult rats than an acute dose regimen, and the RDLMN assay might be capable of detecting genotoxic rodent hepatocarcinogens at dose levels that are typically undetectable in BM MN assays.

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1. Introduction

Many chemicals are metabolized in the liver to yield genotoxins, and many genotoxins can induce liver tumors in rodents [1]. The liver micronucleus (MN) assay is useful for detecting genotoxic rodent hepatocarcinogens, and the Collaborative Study Group for the Micronucleus test (CSGMT) of the Mammalian Mutagenicity Study Group (MMS) of the Japanese Environmental Mutagen Society (JEMS) is presently investigating the utility of the repeated-dose liver micronucleus (RDLMN) assay [2]. The RDLMN assay could contribute to the 3 R principles of the animal welfare, especially a reduction in the number of animals used, and provide comprehensive evaluations, because it can be integrated into repeated-dose general toxicity studies [3,4].

In this study, we evaluated the effectiveness of 14- and 28-day RDLMN assays using monocrotaline (MCT) as the test chemical. MCT is a pyrrolizidine alkaloid that induces tumors in rat livers [1,5] and is genotoxic; it induces the formation of DNA adducts [6] and multiple-fragment DNA crosslinks [7,8]. In a previous study of the bone marrow (BM) MN assay, MCT was reported to produce a negative result with repeated dosing although it produced a positive response with an acute dosing regimen [9]. It was considered that the negative result was due to the lower dose levels as a result of toxicity in the repeated-dose assay [9]. We already know that the LMN assay can be performed effectively with relatively lower dose levels using a repeated-dosing regimen [3], and therefore the ability of MCT to induce MNs in the liver was investigated with both repeated and acute dose regimens. In addition to the LMN assays, the BM MN and peripheral blood (PB) MN assays were conducted in the repeat and acute experiments, respectively, as comparative tests to the LMN assays.

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2. Materials and methods

2.1. Animals

Male Sprague–Dawley (SD) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and were 6-weeks old at the start of the experiments involving the repeated-dose regimens (14 days and 28 days) as outlined in summary report [2]. For the acute dose MN assay, male SD rats were purchased from the same breeder and used for the experiments at the age of 26 days (juvenile rats) as described in the previous report [10] or at the age of 7 weeks (young adult rats), which is the usually used for acute dose MN assays in our test facility. The animals were housed at 22 ± 3 °C and 55 ± 20% relative humidity on a 12 h light/dark cycle and allowed free access to food and drinking water. Five animals were randomly assigned to each treatment group. All animal experimental procedures were performed in accordance with the animal study protocol, which was approved by the Institutional Animal Care and Use Committee of the testing facility.

2.2. Chemicals

Monocrotaline (MCT, CAS no. 315-22-0, >98.0% purity) was purchased from Sigma–Aldrich Corp. (MO, USA), dissolved in water for injection (Japan Pharmacopeia, Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan), and administered by gavage to the animals. For the control, water for injection was administered in the same manner as the test chemical was administered.

2.3. Dose levels and treatment

For the 28 days repeated-dose assay, we initially tested MCT at 3.75, 7.5 and 15 mg/kg/day which were used in the repeated-dose BM and PB MN assay previously [9]. Some of the rats treated at the doses of 7.5 and 15 mg/kg/day died in the initial test; thus, we decided to repeat the assay with the lower doses of 0.15, 0.5 and 1.5 mg/kg/day that were administered once daily for 14 or 28 days (only at the higher two doses, 0.5 and 1.5 mg/kg/day, in the 14-day experiment). In the acute dose assay, we administered 15, 30 and 60 mg/kg/day to juvenile and young adult rats once daily for 2 consecutive days; these doses were shown to induce MN in the BM and PB in the previous report [11]. For the repeated-dose assays, the rats were weighted prior to administration on Days 1, 4, 8, 11 and 15 for the 14-day experiment and Days 1, 4, 8, 11, 15, 18, 22, 25 and 29 for the 28-day experiment. For the acute dose assay, the rats were weighed prior to administration on Days 1 and 2, again on Day 4, and at necropsy on Day 6. For all assays, we anaesthetized the animals with thiopental sodium and euthanized the animals by cutting the abdominal aorta. We administered all doses at a rate of 10 mL per kg body weight per day, and defined the date of the first administration as Day 1.

2.4. Liver assays

In the repeated-dose assays, the animals were euthanized 24 h after the final dosing (Day 15 or 29) following fasting for approximately 18 h. In the acute dose assays, the animals were euthanized on the 4th day after the final dose (Day 6) without fasting. This sampling point in the acute dose assays was reported to be the most appropriate point for detecting the inducibility of MN hepatocytes (HEPs) in juvenile rats treated with double dosing [10]. The HEPs-specimens were prepared and observed following the standard procedure described in the summary paper of the collaborative study [2]. Briefly, approximately 1 g of the liver was sliced into approximately 1-mm thick slices, and the tissue samples were digested with 100 U/mL of collagenase solution (Collagenase S, >820 U/g, Yakult Pharmaceutical Industry, Co., Ltd.) at approximately 37 °C in a water bath for 1 h with shaking. After digestion, the tissues and the collagenase solution were suspended well with a pipette to isolate the HEPs. The isolated HEPs were fixed with 10% formalin. Just prior to microscopic observation, the HEPs was stained with a staining solution containing 500 μg/mL acridine orange (A.O.) and 10 μg/mL 4′6-diamidino-2-phenylindole dihydrochloride (DAPI). The slide specimens were observed under a fluorescent microscope with ultraviolet excitation filter. A total of 2000 parenchymal HEPs were examined for each animal to count the number of micronucleated HEPs (MNHEPs) according to a previously described method [2].

2.5. Bone marrow and peripheral blood assays

The BM was used for the MN assay in the repeated dose experiment. Meanwhile, we used the PB in the acute dose experiment in order to evaluate the MN inducibility using the identical animals for both the liver and hematopoietic tissue simultaneously [10], since the BM MN assays are often difficult to combine with acute dose liver micronucleus assays due to the differences in the appropriate sampling times for the two assays. It was reported that the peripheral blood compartment of rats can be used effectively to detect micronuclei in hematopoietic tissue [12].

For the BM assay in the repeated-dose experiments, after excision of the liver, the femur was excised from each animal and its cavity flushed with 10% formalin to collect the cells. For the PB assay in the acute dose experiments, as recommended in OECD test guideline No. 474, approximately 30 μL blood was collected from the caudal vein 48 hours after the final dosing (Day 4) and suspended in 10% formalin. These fixed BM/PB cell suspensions were stored at room temperature, and were stained with 500 μg/mL A.O. just prior to microscopic observation under fluorescent light with blue light excitation. We examined 2000 immature erythrocytes (IMEs) and counted the numbers of micronucleated IMEs (MNIMEs) for the BM MN assay or examined 2000 RETs and counted the numbers of MNRETs for the PB MN assay. Then the proportion of IMEs or RETs per 1000 erythrocytes was calculated as a parameter of the hematopoietic function of the BM of each animal [2].

2.6. Histopathological examination

For the 14- and 28-day repeated-dose experiments, the liver tissue of the left lateral lobe (with the exception of the portion used for the RDLMN assay) was fixed in 10% formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined histopathologically.

2.7. Statistical analyses

Using the conditional binomial test described by Kastenbaum and Bowman [13] at upper-tailed significance levels of 5% and 1%, the differences in the incidences of MNHEPs, MNIMEs, and MNRETs between the MCT-treated groups and the vehicle control group were analyzed. When a significant difference was detected, a Cochran–Armitage trend test was subsequently conducted to confirm dose response. The other quantitative data, i.e., body weight and ratio of IMEs or RETs among 1000 erythrocytes, were analyzed using multiple comparison tests as follows: homogeneity of variance was examined using the Bartlett's test, and homogeneous or heterogeneous variance was analyzed using the one-way analysis of variance or the Kruskal–Wallis test. When a significant between-group difference was found, Dunnett's test or a Dunnett-type multiple comparison test was used to assess the differences.

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