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Evaluation of a repeated-dose liver micronucleus assay with 2,6-dinitrotoluene using young adult rats

Tadashi Imamura[a,*], Akiko Koeda[a], Kiyoshi Morimoto[a], Hirofumi Hatakeyama[a], Hiroshi Suzuki[a], Yumi Wako[b], Kazufumi Kawasako[b], Koji Otabe[a], Shin-ichi Sato[a]

[a] Ina Research Inc., 2148-188 Nishiminowa, Ina-shi, Nagano 399-4501, Japan
[b] LSI Medience Corporation, 14-1 Sunayama, Kamisu-shi, Ibaraki 314-0255, Japan

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ABSTRACT

As part of a collaborative study by the Mammalian Mutagenicity Study Group of the Environmental Mutagen Society of Japan, we examined micronucleus induction in hepatocytes following oral administration of 2,6-dinitrotoluene (2,6-DNT) at 30, 40, and 50 mg/kg/day for 14 days or at 20, 30, and 40 mg/kg/day for 28 days to young adult male rats. This compound is known to be a rat liver carcinogen. The formation of micronucleated hepatocytes was confirmed to be dose-dependent with statistically significant increases observed in both treatments. In contrast, no statistically significant changes in the percentage of micronucleated immature erythrocytes were observed in any dose group in the bone marrow micronucleus assay. These results indicated that the repeated-dose liver micronucleus assay has the potential to detect genotoxic hepatocarcinogens and can be integrated into general toxicological studies.

1. Introduction

The Mammalian Mutagenicity Study Group, a subgroup of the Environmental Mutagen Society of Japan, evaluated repeated-dose liver and gastrointestinal tract micronucleus assays using young adult rats. This study was performed as a part of the collaborative study.

The liver carcinogen 2,6-dinitrotoluene (2,6-DNT) was selected to demonstrate the utility of the repeated-dose liver micronucleus (RDLMN) assay. This compound induces hepatotoxicity and is classified as a 2B liver carcinogen by the International Agency for Research on Cancer [1]. Dinitrotoluenes, including 2,6-DNT, are used during organic synthesis in the production of toluidines and dyes [1]. Following oral administration of a 2,6-DNT solution to rats, this compound is oxidized by cytochrome P-450 and is excreted in the bile and urine [1,2]. Subsequently, biliary metabolites produced by glucuronidation are reduced by the intestinal flora and reabsorbed from the intestine (enterohepatic circulation). Absorbed metabolites (amines) are then metabolized by *N*-hydroxylation or converted into sulfate conjugates. These unstable metabolites give rise to carbonium and nitrenium ions and can bind covalently to hepatic DNA and protein.

2,6-DNT was found to have a weak clastogenic potential in the *in vitro* chromosomal aberration assay and moderate mutagenic potential in the bacterial reverse mutation assay (Ames test) [3]. In contrast, the result of a bone marrow micronucleus assay was negative [4]. Furthermore, it has been reported that this compound produces positive responses in rat liver in the unscheduled DNA synthesis assay [5], the alkaline single-cell gel electrophoresis (Comet) assay [6], the ^{32}P-postlabelling assay [7], and the young rat liver micronucleus assay [8].

As mentioned above, 2,6-DNT induces DNA damage and micronucleus formation in rat liver. Therefore, this study was performed to confirm the genotoxic potential of 2,6-DNT during repeated-dose administration using the liver micronucleus assay. These assays involved repeated dosing for 14 or 28 days, and focused on micronucleus induction in hepatocytes and bone marrow immature erythrocytes. Concurrently, histopathology of the liver was also performed.

2. Materials and methods

2.1. Animals

No sex-dependent difference in the total amount of the 2,6-DNT metabolites was noted in Fisher 344 rats [1]. Also in the results of the acute toxicity study of 2,6-DNT in Crl:CD(SD) rats, LD_{50} values were almost equal between males and females [9]. Therefore, male Crl:CD(SD) rats were purchased from Charles River Japan Inc. (Atsugi, Japan) and used at 6 weeks of age following a 1-week acclimation period in this study.

* Corresponding author. Tel.: +81 265 73 8611; fax: +81 265 73 8612.
E-mail address: t-imamura@ina-research.co.jp (T. Imamura).

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2.2. Test articles and dosage

2,6-DNT (CAS No. 606-20-2, purity 99.0%) purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) was dissolved in olive oil and dosed at 10 mL/kg body weight. Dosing was performed once a day *via* oral gavage. In the 14-day assay, 50 mg/kg/day was set as the high dose, since significant micronucleus induction was observed in the young rat liver micronucleus assay [8], and 2 lower doses were then set at 40 and 30 mg/kg/day using a common difference of 10 mg/kg/day. In the 28-day assay, 40 mg/kg/day was set as the high dose. As the dosing period is two times longer than that of the 14-day assay, the dose of 40 mg/kg/day was set to minimize the appearance of severe toxicity. Two doses were then set at 30 and 20 mg/kg/day using a common difference of 10 mg/kg/day. Olive oil was administered as the negative control. Five animals were used in each group. The animals were necropsied one day after the last dosing, and histological samples were taken and specimens for the micronucleus assay were prepared. This study was conducted in compliance with the "Act on Welfare and Management of Animals" in Japan, the "Guidance for Animal Care and Use" of Ina Research Inc., and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee (IACUC) of Ina Research Inc., which is fully accredited by AAALAC International (Accredited Unit No. 001107).

2.3. Liver and bone marrow micronucleus assays

Methods used for preparation of the specimens are described briefly below. A detailed description can be found in a summary paper [10]. Approximately 1 g of the lateral left lobe of the liver was sliced at a thickness of 0.5–1 mm, and collagenase digestion solution was added before incubation for 1 h at 37 °C. After incubation, a cell suspension was prepared and fixed with 10% formalin. The hepatocyte specimens were observed under a fluorescent microscope at 400× magnification with U-excitation. Two thousand hepatocytes per animal were observed blindly, and the percentages of micronucleated hepatocytes and mitotic index ratios were calculated.

Bone marrow cells were flushed into tubes from the femur with fetal bovine serum and smear specimens were prepared on glass slides. The specimens were observed under a fluorescent microscope at 1000× magnification with B-excitation. In addition, 2000 immature erythrocytes per animal were observed blindly and the percentages of micronucleated immature erythrocytes were calculated. The percentage of immature erythrocytes, used as an indicator of bone marrow suppression, was also calculated using 1000 erythrocytes.

2.4. Histopathological evaluation

The liver tissue was fixed with 10% formalin and embedded in paraffin following staining with hematoxylin and eosin. Histopathological evaluation was performed under a light microscope.

2.5. Statistical analyses

Differences in micronucleated hepatocytes and micronucleated immature erythrocytes between the negative control and the other groups were analyzed for statistical significance using the conditional binomial test reported by Kastenbaum and Bowman [11].

The body and liver weight data were analyzed for statistical significance by the multiple comparison test (5% level of significance). Homogeneity of variance was examined by Bartlett's test. A one-way analysis of variance was applied when the variance was homogeneous, and Kruskal–Wallis test was applied when the variance was heterogeneous. Statistically significant differences between the groups were assessed by Dunnett's test or Rank test of the Dunnett-type.

3. Results

Liver and bone marrow micronucleus assays were performed following repeated dosing of 2,6-DNT for 14 or 28 days. Consequently, suppression of body weight gain was observed at all doses during both the 14-day and 28-day treatments (Table 1 and Figs. 1 and 2). A statistically significant decrease in the absolute liver weight was noted at 40 mg/kg/day during the 28-day treatment. In contrast, statistically significant increases in the relative liver weight were noted at 30 and 40 mg/kg/day during the 14-day treatment and at 20 and 30 mg/kg/day during the 28-day treatment (Table 1). No deaths or other changes in the general physical condition of any animal in any group were observed during the treatment periods.

Results of the RDLMN assay are summarized in Table 2. A statistically significant, dose-dependent induction of the percentage of micronucleated hepatocytes was observed during both treatment periods. No significant change in mitotic index ratios was observed at any dose levels. The percentages of micronucleated hepatocytes at the same doses in the 14- and 28-day treatments were 0.29% and 0.42%, respectively, at 30 mg/kg/day and 0.36% and 0.69%, respectively, at 40 mg/kg/day. A higher incidence of micronucleated hepatocytes was therefore observed in the 28-day treatment than in the 14-day treatment.

No statistically significant changes in the percentages of immature erythrocytes or micronucleated immature erythrocytes were observed at any dose level in the bone marrow micronucleus assay (Table 2). The percentage of micronucleated hepatocytes in the negative control group of young rats was almost equal to that in the RDLMN assay.

Histopathological findings in the liver following treatment with 2,6-DNT are summarized in Table 3. Inflammatory cell infiltration and diffuse hypertrophy were observed at dose levels of 30 mg/kg/day and above, and oval cell proliferation was observed at all dose levels. While single-cell necrosis was observed at all dose levels in the 14-day treatment, it was not observed at any dose

Table 1
Absolute and relative organ weights of male rats treated with 2,6-DNT.

Dose (mg/kg/day)	14 days			28 days		
	Body weight	Liver		Body weight	Liver	
	(g)	(g)	(g%)	(g)	(g)	(g%)
0						
Mean	307	9.2	3.01	393	12.0	3.05
S.D.	18	0.6	0.03	24	0.9	0.15
20						
Mean	–	–	–	333**	12.0	3.56*
S.D.	–	–	–	33	2.4	0.42
30						
Mean	259*	9.4	3.60#	320**	11.1	3.48*
S.D.	29	1.6	0.26	12	0.9	0.20
40						
Mean	259*	9.6	3.71##	298**	9.8#	3.30
S.D.	25	0.9	0.16	22	0.5	0.12
50						
Mean	256*	8.6	3.35	–	–	–
S.D.	27	1.1	0.30	–	–	–

* $P < 0.05$, significantly different from negative control (Dunnett's test).
** $P < 0.01$, significantly different from negative control (Rank test of Dunnett type).
\# $P < 0.05$, significantly different from negative control (Rank test of Dunnett type).
\## $P < 0.01$, significantly different from negative control (Rank test of Dunnett type).