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In Vivo Micronucleus Test of Methylcyclopentane and 1,4-Dichlorobutane

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We investigated the genotoxicity of two chemicals, methylcyclopentane and 1,4-dichlorobutane with in vivo micronucleus test. Although these two chemicals have already been tested many times, a micronucleus test has not been conducted and the usage of these chemicals has been recently increased. 7 week male ICR mice were tested at dosages of 500, 1,000, and 2,000 mg/kg for methylcyclopentane and 500, 1,000, and 2,000 mg/kg for 1,4-dichlorobutane, respectively. After 24 hours of oral administration with the two chemicals, the mice were sacrificed and their bone marrow cells were prepared for smearing slides. As a result of counting the micronucleated polychromatic erythrocyte (MNPCE) of 2,000 polychromatic erythrocytes (PCE), all treated groups expressed no statistically significant increase of MNPCE compared to the negative control group. There were no clinical signs related with the oral exposure of these two chemicals. It was concluded that the two chemicals did not induce micronucleus in the bone marrow cells of ICR mice, and there was no direct proportion with dosage. These results indicate that the two chemicals have no mutagenic potential under each study condition.

Key words: Bone marrow, Methylcyclopentane, Micronucleus test, 1,4-Dichlorobutane

1. Introduction

The necessity for a chemical hazard assessment has been increased because the number of workers exposed to chemicals has risen with the development of chemical industries. The *in vivo* micronucleus test was performed on mammalian bone marrow cells treated with two chemicals of methylcyclopentane (CAS No. 96-37-7) and 1,4-dichlorobutane (CAS No. 110-56-5), the definitive information of which is insufficient. The toxicological information on these two chemicals as gained in this study will be used to promote worker's "rights to know", and to prepare or update the material safety data sheet (MSDS) for these chemicals.

Methylcyclopentane is classified to a flammable liquid (Category 2), acute toxicity-oral (Category 4), skin irritation (Category 2), eye irritation (Category 2), specific target organ toxicity - single exposure (Category 3), aspiration hazard (Category 1) and highly flammable. It is insoluble in water, miscible in ethanol, ether, and acetone. But to the best of our knowledge, the chemical, physical, and toxicological properties have not been thoroughly investigated[1)].

Polyneuropathy of the motor type was reported by workers in a handicraft firm. The solvent was analyzed and showed the presence of methylcyclopentane. The neurotoxicity of methylcyclopentane and neurotoxic interaction with other compounds has yet to be determined[2)]. National Institute for Occupational Safety and Health has statistically estimated that 1,053 workers are potentially exposed to methylcyclopentane in the USA. The exposure to methylcyclopentane will occur through ingestion and inhalation[3)].

The average concentration of methylcyclopentane in the air from gasoline vapors, to which petroleum industry workers may be exposed, for outside operators

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is 0.148 mg/m^3, for transport drivers is 0.560 mg/m^3, and for service attendants is 0.763 mg/m^3 [4].

Methylcyclopentane has been identified as a volatile component of baked potatoes and beef, and was qualitatively found in samples of mother's milk[5].

1,4-Dichlorobutane is clear, colorless liquid classified to a flammable liquid (Category 3), specific target organ toxicity - repeated exposure (Category 2) and has irritating to eyes, respiratory system and skin. And it has a melting point of -38°C, a boiling point 161 - 163°C, a flash point 40°C - closed cup and a density 1,16 g/ml at 25°C[6].

It was evaluated that methylcyclopentane and 1,4-dichlorobutane are non-mutagenic by bacterial reverse mutagenic assays with Salmonella typhimurium and Escherichia coli with or without metabolic activation, but all of them are non-GLP tests[7,8], and the available genotoxicity data on these two chemicals are still controversial with and without mammalian metabolic activation (S9)[9]. So it was necessary for further research according to GLP guideline to secure quality assurance of the test.

As the above, many tests have been conducted other than the micronucleus test. The purpose of this micronucleus induction is to screen the cytogenetic damage that results in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes.

2. Materials and Methods

2.1 Chemicals and animal feeding conditions

Methylcyclopentane (Sigma, St Louis MO, USA, Lot No. 00413EJ, 97%) and 1,4-dichlorobutane (Sigma, St Louis MO, USA, Lot No. 1323541, 99%) were used as the test chemicals. Olive oil (Sigma, St Louis MO, USA, Lot No. 058K0684) was used as a solvent according to the results of the solubility test. Mitomycin C (MMC) (Sigma, St Louis MO, USA, Lot No. 028K1815) was used as a positive control.

2.2 Animals and experimental design

The mouse bone marrow micronucleus test was carried out according to OECD guidelines (TG 474)[10]. It was performed using 7 week-old male ICR mice (7 weeks old, Japan SLC Inc., Shizuoka, Japan) at 500, 1,000, and 2,000 mg/kg dosages with methylcyclopentane and 1,4-dichlorobutane, respectively. Test was performed at 24 hours after treatment with the two chemicals administered orally. Each group was consisted of 6 male mice. The animal studies were approved by an animal ethics committee to ensure that appropriate animal care before the animals was obtained for research.

2.3 Bone marrow preparation and micronucleus test

Bone marrow cells were obtained from the femurs immediately following sacrifice. The bone marrow was flushed from the femurs and spread onto slides. The slides were air-dried, fixed with ethanol, and stained with a fluorescent DNA specific stain that easily illuminates any micronuclei that may be present. The 2,000 polychromatic erythrocytes (PCEs, reticulocytes; immature erythrocytes) were scored per animal for the frequency of micronucleated cells in each of the 6 animals per dosage group. In addition, the percentage of PCEs among the 500 erythrocytes in the bone marrow was scored for each dosage group as an indicator of chemical-induced toxicity.

The presence of micronucleated polychromatic erythrocytes was visually scored (at least 2,000 cells per mouse) by optical microscopy using a fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) with a BA-2 filter.

2.4 Evaluation and data analysis

The final conclusion for a micronucleus test was determined in consideration of the results of the statistical analyses.

The experimental and control micronucleus frequency for each specimen within and between the different mice strains were compared with the one way Analysis of Variance (ANOVA, $P<0.001$) test and the Dunnett's Method ($P<0.05$) using the SigmaStat v. 3.11.

3. Results

3.1 Frequencies of micronucleus induction and cytotoxicity

The preliminary tests were performed as a limit test to determine the maximum dosage. The inhibition of proliferation in the bone marrow cells was not observed in these tests for the two chemicals.

The frequencies of erythrocytes with micronucleus induction were 0.26±0.11%, 0.13±0.11%, 0.26±0.16%, and 0.34±0.17% in the negative control group, 500, 1,000, and 2,000 mg/kg in the methylcyclopentane treated group, respectively.

The ratios of PCEs within total erythrocytes were 46.95±7.66%, 42.04±9.64%, 47.34±7.58%, and 43.55± 6.87% in the negative control group, 500, 1,000, and 2,000 mg/kg in the methylcyclopentane treated group, respectively.

Statistically significant changes were not observed compared with the negative control group (Table 1).

The frequencies of erythrocytes with micronucleus induction were 0.22±0.16%, 0.09±0.08%, 0.07±0.05%, and 0.09±0.05% in the negative control group, 500, 1,000, and 2,000 mg/kg in the 1,4-dichlorobutane treated group, respectively.

The ratio of PCEs (polychromatic erythrocytes) within total erythrocytes were 38.17±6.62%, 38.07± 9.50%, 41.46±8.26%, and 41.69±8.72% in the negative control group, 500, 1,000, and 2,000 mg/kg in the 1,4-dichlorobutane treated group, respectively. There were no statistically significant changes observed compared with the negative control group (Table 2).

4. Discussion

Micronuclei were first used to quantify chromosomal damage by Scott and Evans[11] in the root tips of broad beans, *Viciafaba*. The assay is now recognized as one of the most successful and reliable assays for genotoxic carcinogens, so this assay was used in this study with

Table 1. Results of the main micronucleus test with methylcyclopentane (for 24 hr)

Groups	PCE* observed	MNPCE** observed	MNPCE frequency (%)	(PCE+NCE***) counted	PCE counted	PCE/(PCE+NCE) (%)
Negative control	2085±49.04	5.5± 2.35	0.26±0.11	519±15.02	243.50±38.65	46.95±7.66
500 mg/kg b.w.	2096±68.00	2.67± 2.34	0.13±0.11	527.50±13.03	221.83±51.32	42.04±9.64
1,000 mg/kg b.w.	2072.5±49.24	5.34± 3.14	0.26±0.16	518.17±10.26	245.33±40.50	47.34±7.58
2,000 mg/kg b.w.	2090±36.56	7.17± 3.60	0.34±0.17	523.50± 7.94	227.67±33.42	43.55±6.87
Positive control	2053.17±29.68	42.67±16.73	2.08±0.83	533.17±15.04	176±37.23	33.17±7.80

*PCE; Polychromatic erythrocyte
**MNPCE; Micronucleated Polychromatic erythrocyte
***NCE; Normochromatic erythrocyte
Compared with negative control $P<0.05$
All values are expressed as mean±S.D.

Table 2. Results of the main micronucleus test with 1,4-dichlorobutane (for 24 hr)

Groups	PCE* observed	MNPCE** observed	MNPCE frequency (%)	(PCE+NCE***) counted	PCE counted	PCE/(PCE+NCE) (%)
Negative control	2112.84±76.15	4.5±3.33	0.22±0.16	515.34±10.17	196.67±34.09	38.17± 6.62
500 mg/kg b.w.	2105±85.57	1.83±1.72	0.09±0.08	515.83±17.31	197.17±53.36	38.07± 9.50
1,000 mg/kg b.w.	2078.5±39.53	1.5±1.05	0.07±0.05	532.83±28.51	220.33±41.97	41.46± 8.26
2,000 mg/kg b.w.	2116.5±61.15	1.83±0.98	0.09±0.05	537.50±27.88	223.83±46.05	41.69± 8.72
Positive control	2096.33±45.23	22.17±8.04	1.05±0.36	518.17±10.15	225.83±62.54	43.55±11.78

*PCE; Polychromatic erythrocyte
**MNPCE; Micronucleated Polychromatic erythrocyte
***NCE; Normochromatic erythrocyte
Compared with negative control $P<0.05$
All values are expressed as mean±S.D.

methylcyclopentane and 1,4-dichlorobutane.

Commercial hexane is a solvent consisted of six-carbon isomers principally n-hexane (53%), 3-methyl pentane (16%), methylcyclopentane (14%), and 2-methyl pentane (12%). Male and female SD (Sprague-Dawley) rats were exposed to commercial hexane vapor at target concentrations of 0, 900, 3,000, or 9,000 ppm for 6 hr/day, 5 days/week, over two generations. At both the F0 breed to produce Fl litters and the Fl breed to produce F2 litters, reproductive parameters were unaffected by commercial hexane exposure. Litter size and postnatal survival were not significantly different between exposure groups. However, reductions in body weight and body weight gain were observed in both Fl and F2 litters exposed to 9,000 ppm. Effects on body weight were not observed in offspring exposed to the two lower concentrations of commercial hexane. The exposure of rats to commercial hexane for two generations resulted in reduced body weight gains at 9,000 ppm but no adverse effects on reproduction. The NOEL for this study was 3,000 ppm[12].

Methylcyclopentane's production and use in organic synthesis, as an extractive solvent, and may result in its release to the environment through various waste streams. Methylcyclopentane has been identified in numerous environmental samples including emissions from automobiles, drinking water, surface water, industrial effluents, atmospheric samples, and sediments. It has also been detected as an indoor pollutant from various household products, as volatile component of baked potatoes and beef, in mother's milk and in human expired air. Methylcyclopentane was detected in the expired air from 4 out of 8 subjects (all male: 2 smokers and 2 non-smokers) at levels of 0.98 and 0.027 μg/h for the non-smokers and 11.0 and 0.31 μg/h for the smokers[13].

1,4-dichlorobutane is not listed by ACGIH, IARC or NTP, it was evaluated as a non-mutagenic by bacterial reverse mutagenic assays with *Salmonella typhimurium* and *E. coli* with or without metabolic activation as non-GLP tests[8]. So it was necessary for further research with it to secure quality assurance of the test.

Micronucleus (MN) formation is either from chromosome breakage (clastogenicity) or aneuploidy. By using pancentromeric probes, it is possible to draw conclusions if MN is formed as a consequence of chromosomal breakage (clastogenicity) or aneuploidy.

Some authors have described sex as an important variable in the micronucleus test[14], with males generally more sensitive to the induction of micronuclei than females. However, other studies have shown no sex-related differences in micronucleus test results[15].

Based on this study, we suggest that future studies be directed toward chronic inhalation, carcinogenic tests, and so on. It is suggested that further investigations, such as Fragment Length Analysis using Repair Enzymes (FLARE) assay, Comet assay with repair enzyme as Fpg, Endo III, and real time Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and other developing tools, be performed[16]. Further, performing these tests with many other chemicals would be useful as a biomarker for chemical risk assessment.

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