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Evaluation of the Genetic Toxicity of Synthetic Chemicals (XIV) -*In vivo* Bone Marrow Micronucleus Assay of 11 Synthetic Chemicals in Mice-

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합성화학물질들의 유전독성평가(XIV)-마우스의 골수세포를 이용한 11종 합성화학물질들의 생체내 소핵시험-

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요 약

합성화학물질들이 환경으로의 유입은 인체에는 물론 환경생태계에 많은 영향을 미치므로 이들의 유해성 검증은 매우 중요한 일이라 할 수 있다. 실제 산업체에서 사용되는 수많은 화학물질들의 유전적 손상 유발유무는 유해성검증에서 무엇보다 중요한 일이라 할 수 있다.

이에 산업체 공정과정에서 널리 사용되는 것으로 알려진 11종의 합성화학물질에 대해 마우스의 골수세포를 이용한 *in vivo* 소핵시험을 수행하여, 소핵형성 유발유무를 관찰하였다. 양성대조군으로 사용된 mitomycin C는 음성대조군과 비교시 유의하게 소핵을 유발하는 반면, 비교적 마우스에서 높은 50% 치사량을 보이는 thiourea, 2,4-dichlorophenol 및 2,4-toluene diisocyanate 등의 합성물질들을 비롯한 나머지 8종의 물질들은 본 실험결과 통계적으로 유의하게 소핵을 유발하지 않는 것을 관찰 할 수 있었다.

Key words : genotoxicity, clastogenicity, mouse bone marrow, micronucleus

INTRODUCTION

It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that mutagens are carcinogens (McCann *et al.*, 1975) and, for at least some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1977). Several assay systems such as reversion test with bacterial gene mutation (Ames *et al.*, 1975; Maron and Ames, 1983), chromosomal aberration assay with mammalian cells (Ishidate and Odashima, 1977; Ryu *et al*, 1994, 1996a, 2001a, 2002a,b, 2003a), mouse lymphoma tk (+/-) gene assay with L5178Y tk+/- mouse lymphoma cells (Clive *et al.*, 1995; Garriott *et al.*, 1995; Ryu *et al*, 1999), micronucleus assay with rodents (Schmid, 1975; Hayashi *et al.*, 1990, 1992; MacGregor *et al.*, 1990; Tice *et al.*, 1990; Ryu *et al.*, 1996b, 1998,

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2001b, 2003b,c, 2004) have been introduced and also frequently adopted as methods for an index of genotoxicity in worldwide.

Cytogenetic studies on mammalian cells *in vivo* as well as *in vitro* have been introduced as a screening method for DNA-attacking substances. Among several genotoxicity assays, the micronucleus assay using immature bone marrow erythrocytes of mice has been widely used as a simple and sensitive short-term screening method *in vivo* for determining the clastogenicity of chemical substances (Heddle, 1973; Schmid, 1975). As this assay uses "whole animals", it has the merits of including such factors as absorption, distribution, and metabolism of the chemical substances in the evaluation. Although mouse bone marrow young erythrocytes (polychromatic erythrocytes) have most frequently been used as experimental material, young erythrocytes in mouse peripheral blood are increasingly being used as alternative target cells (Hayashi *et al.*, 1990, 1992, 1994; MacGregor *et al.*, 1990; Tice *et al.*, 1990).

In this study, we aim to elucidate the calstogenicity of 11 synthetic chemicals used in chemical process using *in vivo* mouse bone marrow micronucleus assay.

MATERIALS AND METHODS

The experiment was performed as described by Schmid (1975) with some minor modifications (Ryu *et al.*, 1996b, 1998, 2001b, 2003b,c, 2004) which are briefly summarized as follows.

1. Animals and Reagents

Outbred male mice of strain ICR were purchased from Dae-Han Laboratory Animal Co. (Eumsung-gun, Korea) at 7~8 weeks of age. The mice were allowed an adaptation period of about 1 week, then randomized and subjected to the study. Mice were fed commercial pellets and tap water *ad libitum* throughout the acclimation and experiment periods.

Fetal bovine serum (FBS) and Giemsa stain solution were purchased from Gibco-BRL (Gaithersburg, USA). Sodium phosphate and Mitomycin C (MMC; Cat No. M0503) were purchased from Sigma (St. Louis, Mo). Carboxylmethyl cellulose sodium salt (CMC) was purchased from Showa (Japan). MMC was dissolved in physiological saline and injected once intraperitoneally at dose level of 2 mg/kg body weight. The six animals were housed for each group.

2. *In vivo* bone marrow micronucleus assay after intraperitoneal or oral administration in mice

The test article was applied intraperitoneally or orally in three doses in volumes of 10 mL/kg. The test substance was given once, and then 24 hr interval, they killed by cervical dislocation. Normally, the tested dose range included the span from no effect up to complete halt of bone marrow proliferation. Preparation of bone marrow and staining were carried out according to the method worked out Schmid (1975). From the freshly killed animal both femora after 24 hr intraperitoneal or oral administration were removed *in toto*, which means that one was cutting through pelvis and tibia. The bones were then freed from muscle by the use of gauze and fingers. With the needle of appropriate size mounted, about 1 mL of serum was pulled from the tube into a disposable plastic syringe. Then the needle (24 gauge) was inserted a few mm into the proximal part of marrow canal to flush the marrow cells. After centrifugation, the supernatant was removed, and cell pellet suspension of bone marrow cells was dropped onto glass slides, and then air dried. After fixation in methanol, slides were stained with 4% Giemsa in 1/15 M sodium phosphate buffered saline (PBS, pH 6.8) for 30 min, washed with PBS, and then air dried for microscopic observation.

In scoring the preparations, micronuclei were counted in polychromatic and, separately in normochromatic erythrocytes. The rate of micronucleated cells, expressed in percentage, were based on the

total of polychromatic erythrocytes present in the scored optic fields. This mode of scoring, which must always be followed where the test substance markedly influences the proliferation rate in the bone marrow, prevents a distortion of the results by the influx of peripheral blood into the damaged marrow. The scoring of micronucleated normocytes not only serves to recognize the presence of artifacts (which is rare in preparations from mouse) but provides additional interesting information on the mode of action of the test substance. Generally, an incidence of more than 1 micronucleated normocyte per thousand polychromatic erythrocytes indicates an effect on cell stages past the S-phase.

RESULTS AND DISCUSSION

The mouse bone marrow micronucleus assay is based on the detection of the small nucleus (micronucleus) formed from chromosomal damage by chemical substances (Heddle, 1973; Schmid, 1975). The formed micronuclei remain in the cytoplasm. These micronuclei are formed by clastogenic substances and spindle poisons. When the forming function of the spindle body is obstructed, a micronucleus occurs with one to several chromosomes. Therefore, whole chromosomes containing micronuclei are observed as large size fragments rather than lagging chromosome fragments (Yamamoto and Kikuchi, 1980).

Besides, the International Agency for Research on Cancer (IARC) issued monographs containing lists of substances that cause cancer in humans (IARC, 1987). To assess the correlation between the micronucleus induction potency and carcinogenic activity, the micronucleus assay was performed as the collaborative study group for the micronucleus test by the Japanese environmental mutagen society (Morita *et al.*, 1997). The experimental results of the micronucleus assay were evaluated by comparing with published data on the IARC carcinogens. The positive rates for groups 1, 2A and 2B were 68.6, 54.5 and 45.6%, respectively. After incorporating information on the structure-activity relationship, the positive rates of the micronucleus assay became 90.5, 65.2 and 60.0% for IARC groups 1, 2A and 2B, respectively. It must be noted that the positive rates tended to be higher in carcinogens with a higher risk for human carcinogenicity. Based upon these results, it is suggested that the use of the micronucleus assay is useful as an *in vivo* short-term screening method to predict the human carcinogenicity of chemical substances.

The chemical name, CAS number and manufacturer of test chemicals in mice were listed in Table 1, LD_{50} values of test chemicals in mice depending on the administration routes were listed in Table 2. Among the many synthetic chemicals used in chemical reaction processes in industry, thiourea (CAS No. 62-56-6) used as animal glue liquifiers, phorographic fixing agent and silver tarnish removers, acetonitrile (CAS No. 75-05-8) and 2,4-dichlorophenol (CAS No. 120-83-2) used as starting material in organic synthesis. Ethyl methacrylate (CAS No. 97-63-2) is a base material for coating and adhesives, and also used as a chemical intermediate in organic synthesis. 4,4′-Methylene dianiline (CAS No. 101-77-9) and 2,4-toluene diisocyanate (CAS No. 584-84-9) used as chemical intermediate for preparation of polyurethane foams. Dicyclohexyl amine (CAS No. 101-83-7) and bis(2-methoxyethyl) ether (CAS No. 111-96-6) used as corrosion inhibitor and reaction medium for Grignard synthesis, respectively. p-Dioxane (CAS No. 123-91-1) used as stabilizer in chlorinated solvents, and diallyl phthalate (CAS No. 131-17-9) used in critical electrical/electronic applications and in the processing of thermosetting plastics. And also, 2-nitroaniline (CAS No. 88-74-4) use as a chemical intermediate for dyes and dyestuffs. Nevertheless of the diverse and extensive uses of these chemicals in industry, however, there has been few attention to evaluate the genotoxicity of some chemicals.

In this study, we used 7-weeks-old male ICR mice. First of all, we determined the bone marrow sampling time after oral or intraperitoneal treatment

Table 1. List of 11 chemicals for mouse bone marrow micronucleus assay

	Chemical name	CAS No.	Cat. No.	Manufacturer
1.	Thiourea	62-56-6	T8656	S
2.	Acetonitrile	75-05-8	A998-4	F
3.	Ethyl methacrylate	97-63-2	055-01476	W
4.	4,4′-Methylene dianiline	101-77-9	M0288	S
5.	Dicyclohexyl amine	101-83-7	D3003	S
6.	Bis(2-methoxyethyl) ether	111-96-6	M8132	S
7.	2,4-Dichlorophenol	120-83-2	D6023	S
8.	p-Dioxane	123-91-1	D9553	S
9.	diallyl phthalate	131-17-9	010-01483	W
10.	2,4-toluene diisocyanate	584-84-9	T6889	S
11.	2-nitroaniline	88-74-4	N0118	T

S : Sigma-Aldrich Korea, Seoul, Korea F : Fisher Scientific, PA. USA
W : Wako Pure Chemical Industries, Ltd. Osaka, Japan
T : Tokyo Kasei Kogyo Co. Ltd., Japan

Table 2. 50% Lethal dose (LD_{50}) of 11 synthetic chemicals

	Chemical name	Route-animal	LD_{50} (mg/kg)	References
1.	Thiourea	ipr.-mus.	100	EPA(NTP)
2.	Acetonitrile	orl.-mus.	269	HSDB
3.	Ethyl methacrylate	orl.-mus.	7,836	HSDB
4.	4,4′-Methylene dianiline	orl.-mus.	745	RTECS
5.	Dicyclohexyl amine	orl.-mus.	500	RTECS
6.	Bis(2-methoxyethyl) ether	orl.-mus.	3,200 3,700	Our Data RTECS
7.	2,4-Dichlorophenol	ipr.-mus.	153	ME
8.	p-Dioxane	orl.-mus.	5,700	RTECS
9.	diallyl phthalate	ipr.-mus.	700	HSDB
10.	2,4-toluene diisocyanate	ipr.-mus.	67	Our Data
11.	2-nitroaniline	orl.-mus.	1,070	RTECS

RTECS : Registry of Toxic Effects of Chemical Substances
HSDB : Hazardous Substances Data Bank
EPA (NTP) : EPA National Toxicology Program, Acute Hazard Rankings
ME : Ministry of Environment, Korea
ipr.-mus. : intraperitoneal-mouse
orl.-mus. : oral-mouse

of 24 hr or 48 hr interval as indicated in Table 3. The administration dose for each experiment was adopted with half dose of LD_{50} value as the highest dose. The LD_{50} values of bis (2-methoxyethyl) ether (CAS No. 111-96-6) and 2,4-toluene diisocyanate (CAS No. 584-84-9) were determined as 3,200 and 67 mg/kg, respectively, by Lorke method (1983) in our laboratory (Table 2). The positive control, mitomycin C (2 mg/kg, i.p.) revealed significant induction ratio of percentage of micronucleated polychromatic erythrocytes/1,000 polychromatic erythrocytes (MNPCE %/PCE) compared to solvent control. The constant range of ratio percentage of polychromatic erythrocytes/1,000 erythrocytes was also observed in all experiments as summarized in Table 4. The chemicals with relatively high LD_{50} value such as thiourea (CAS No. 62-56-6), 2,4-dichlorophenol (CAS No. 120-83-2) and 2,4-toluene diisocyanate (CAS No. 584-84-9) revealed no significant induction of micronucleated polychromatic erythrocytes in mice.

Table 3. Determination of bone marrow sampling time of 11 chemicals

Test chemicals (CAS No.)	Dose (mg/kg)	Route	Sampling time (hr)	MNPCE %/PCE[a] (Mean±SD)	Ratio % of PCE/PCE+NCE[b](Mean±SD)	*P*-value[c]
Thiourea	50	i.p.	24	0.15±0.09	0.48±0.04	>0.05
(62-56-6)	50		48	0.25±0.05	0.49±0.03	>0.05
Acetonitrile	134.5	p.o.	24	0.15±0.05	0.48±0.01	>0.05
(75-05-8)	134.5		48	0.15±0.10	0.48±0.03	>0.05
Ethyl methacrylate	2,000	p.o.	24	0.27±0.08	0.52±0.02	>0.05
(97-63-2)	2,000		48	0.15±0.09	0.49±0.01	>0.05
4,4′-Methylene dianiline	373	p.o.	24	0.29±0.13	0.48±0.06	>0.05
(101-77-9)	373		48	0.27±0.08	0.63±0.04	>0.05
Dicyclohexyl amine	250	p.o.	24	0.09±0.03	0.48±0.02	>0.05
(101-83-7)	250		48	0.09±0.08	0.45±0.06	>0.05
Bis(2-methoxyethyl) ether	1,600	p.o.	24	0.20±0.10	0.52±0.03	>0.05
(111-96-6)	1,600		48	0.20±0.09	0.47±0.07	>0.05
2,4-Dichlorophenol	153	i.p.	24	0.08±0.06	0.47±0.04	>0.05
(120-83-2)	153		48	0.05±0.05	0.53±0.01	>0.05
p-Dioxane	2,000	p.o.	24	0.02±0.03	0.49±0.05	>0.05
(123-91-1)	2,000		48	0.13±0.06	0.47±0.05	>0.05
diallyl phthalate	350	i.p.	24	0.10±0.05	0.54±0.04	>0.05
(131-17-9)	350		48	0.07±0.03	0.49±0.06	>0.05
2,4-toluene diisocyanate	33.6	i.p.	24	0.18±0.10	0.43±0.07	>0.05
(584-84-9)	33.6		48	0.13±0.06	0.37±0.04	>0.05
2-nitroaniline	535	p.o.	24	0.07±0.06	0.50±0.01	>0.05
(88-74-4)	535		48	0.05±0.05	0.51±0.01	>0.05

[a]MNPCE %/PCE : Percentage of micronucleated polychromatic erythrocytes/2,000 polychromatic erythrocytes

[b]PCE/PCE+NCE : Polychromatic erythrocytes/1,000 erythrocytes

[c]Pairwise comparison to corresponding control, significant at $P<0.05$

From this results, 11 synthetic chemicals used in industry widely have revealed no significant micronucleus induction of clastogenicity in mice in this experiment (Table 4).

Recently, *in vivo* supravital micronucleus assay with peripheral reticulocytes by using acridine orange fluorescent staining (Hayashi *et al.*, 1990, 1992; Heo *et al.*, 1997; Ryu *et al.*, 1998, 2001b) was introduced instead of mouse bone marrow micronucleus assay to distinguish immature erythrocytes in the peripheral bloods. This assay, briefly, the use of a DNA specific stain, acridine orange can eliminate some of the artifacts associated with using a non-DNA specific stain, Giemsa. The immature bone marrow erythrocytes enter circulation in the peripheral blood. Peripheral blood cells are obtained from the tail vein or other appropriate vessel and immediately stained supravitally. By using peripheral blood, the safety evaluation of chemical substances may be expanded from mice to rats or even to humans. As in the peripheral blood assay, the same animal can be used for several sampling and it may be possible to limit the number of animals used and the amount of each substance to be tested. As a result, more useful information about micronucleus induction could be obtained compared to the bone marrow micronucleus assay, and this method using animal blood can be applied to humans, fish, shellfish and also to insects (Hayashi *et al.*, 1998; Tanisho *et al.*, 1998; Peace and Succop, 1999; Saotome *et al.*, 1999). Moreover, a molecular cytogenetic method, *i.e.*, "fluorescent in site hybridization (FISH)", with centromere DNA-probes were developed (Becker *et al.*, 1990; Miller *et al.*, 1991). By this method, the presence of centromeres in micronuclei can be clearly detected, and the ability to detect differences between the micronucleus induced by clastogens or by spindle poisons became possible (Komae *et al.*,

Table 4. *Micronucleus data on the bone marrow of ICR male mice administered i.p. or p.o. with 11 chemicals*

Test chemicals (CAS No.)	Dose (mg/kg)	Route	Sampling time (hr)	MNPCE %/PCE[a] (Mean±SD)	Ratio % of PCE/PCE+NCE[b](Mean±SD)	*P*-value[c]
CMC[d]	0	i.p.	48	0.07±0.04	0.54±0.04	-
Thiourea	50	i.p.	48	0.20±0.08	0.49±0.02	>0.05
(62-56-6)	25		48	0.21±0.14	0.51±0.02	>0.05
	12.5		48	0.22±0.13	0.51±0.04	>0.05
Saline	0	p.o.	24	0.30±0.09	0.48±0.03	-
Acetonitrile	134.5	p.o.	24	0.35±0.13	0.50±0.03	>0.05
(75-05-8)	67.3		24	0.12±0.06	0.47±0.06	>0.05
	33.7		24	0.26±0.08	0.51±0.02	>0.05
Saline	0	p.o.	24	0.30±0.09	0.48±0.03	-
Ethyl methacrylate	2,000	p.o.	24	0.21±0.10	0.53±0.02	>0.05
(97-63-2)	1,000		24	0.24±0.08	0.54±0.01	>0.05
	500		24	0.20±0.06	0.56±0.05	>0.05
CMC	0	p.o.	24	0.18±0.12	0.49±0.02	-
4,4′-Methylene dianiline	373	p.o.	24	0.25±0.10	0.49±0.05	>0.05
(101-77-9)	186.5		24	0.18±0.04	0.52±0.03	>0.05
(101-77-9)	93.3		24	0.24±0.08	0.53±0.01	>0.05
Corn oil	0	p.o.	24	0.16±0.07	0.48±0.05	-
Dicyclohexyl amine	250	p.o.	24	0.09±0.03	0.48±0.02	>0.05
(101-83-7)	125		24	0.19±0.08	0.52±0.03	>0.05
	62.5		24	0.21±0.13	0.51±0.02	>0.05
Saline	0	p.o.	24	0.30±0.09	0.48±0.03	-
Bis(2-methoxyethyl)	1,600	p.o.	24	0.28±0.14	0.46±0.06	>0.05
ether	800		24	0.22±0.06	0.51±0.03	>0.05
(111-96-6)	400		24	0.20±0.09	0.51±0.03	>0.05
Corn oil	0	i.p.	24	0.12±0.10	0.51±0.03	-
2,4-Dichlorophenol	153	i.p.	24	0.08±0.04	0.47±0.03	>0.05
(120-83-2)	77		24	0.08±0.06	0.48±0.06	>0.05
	39		24	0.07±0.03	0.57±0.02	>0.05
Saline	0	p.o.	48	0.30±0.09	0.48±0.03	-
p-Dioxane	2,000	p.o.	48	0.10±0.06	0.53±0.05	>0.05
(123-91-1)	1,000		48	0.15±0.16	0.51±0.05	>0.05
	500		48	0.06±0.02	0.51±0.04	>0.05
Corn oil	0	i.p.	24	0.10±0.11	0.49±0.02	-
diallyl phthalate	350	i.p.	24	0.06±0.07	0.48±0.06	>0.05
(131-17-9)	175		24	0.07±0.07	0.50±0.07	>0.05
	87.5		24	0.06±0.06	0.48±0.02	>0.05
Corn oil	0	i.p.	24	0.18±0.12	0.49±0.02	-
2,4-toluene diisocyanate	33.6	i.p.	24	0.14±0.11	0.48±0.10	>0.05
(584-84-9)	16.8		24	0.16±0.07	0.55±0.06	>0.05
	8.4		24	0.16±0.04	0.56±0.05	>0.05
CMC	0	p.o.	24	0.07±0.01	0.49±0.03	-
2-nitroaniline	535	p.o.	24	0.04±0.05	0.50±0.01	>0.05
(88-74-4)	267.5		24	0.02±0.03	0.51±0.02	>0.05
	133.8		24	0.02±0.03	0.55±0.03	>0.05
MMC[e]	2	i.p.	24	3.49±0.78*	0.46±0.01	<0.01
	2	i.p.	48	1.71±1.04**	0.47±0.01	<0.01

[a]MNPCE %/PCE : Percentage of micronucleated polychromatic erythrocytes/2,000 polychromatic erythrocytes

[b]PCE/PCE+NCE : Polychromatic erythrocytes/1,000 erythrocytes

[c]Pairwise comparison to corresponding control, significant at $P<0.05$ [d]CMC : Carboxylmethyl cellulose Na salt

[e]MMC : Mitomycin C, * : n=9, ** : n=2

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