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# Evaluation of the Genetic Toxicity of Synthetic Chemicals (VIII)
## – *In vivo* Bone Marrow Micronucleus Assay of 8 Synthetic Chemicals in Mice –

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# 합성화학물질들의 유전독성평가(VIII)
## –마우스의 골수세포를 이용한 8종 합성화학물질들의 생체내 소핵시험–

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**요 약**

합성화학물질들이 환경중에 유입되어 인체에는 물론 환경생태계에 많은 영향을 미치고 있어 이들의 유해성 검증은 매우 중요한 일이라 할 수 있다. 실제 산업체에서 사용되는 수많은 화학물질들의 유전적 손상 유발유무는 더욱이 중요한 일이라 할 수 있다.

이에 산업체 공정과정에서 널리 사용되는 것으로 알려진 8종의 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 phenylisocyanate, m-aminochlorobenzene 및 2-chloro-4-nitroaniline 등의 합성물질들을 비롯한 나머지 5종의 물질들은 본 실험결과 통계적으로 유의하게 소핵을 유발하지 않는 것을 관찰 할 수 있었다.

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

## INTRODUCTION

Cytogenetic studies on mammalian cells *in vivo* as well as *in vitro* have been introduced as a screening method for DNA-attacking substances. 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.*, 1973, 1975; Maron and Ames, 1983), chromosomal aberration assay with mammalian cells (Ishidate and Odashima, 1977; Ryu *et al.*, 1993, 1994, 1996a, 2001a, 2002a, b), mouse lymphoma tk(+/−) gene assay with L5178Y tk+/- mouse

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lymphoma cells (Clive *et al.*, 1983, 1995; Sawyer *et al.*, 1985; Garriott *et al.*, 1995; Oberly and Garriott, 1996; Ryu *et al.*, 1999), micronucleus assay with rodents (Hayashi *et al.*, 1982, 1990, 1992; MacGregor *et al.*, 1980, 1990; Tice *et al.*, 1990; Schmid, 1975; Ryu *et al.*, 1996b, 1998, 2001b) have been introduced and also frequently adopted as methods for an index of genotoxicity in worldwide.

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 (Schmid, 1975; Heddle, 1973). 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 (MacGregor *et al.*, 1980, 1990; Tice *et al.*, 1990; Hayashi *et al.*, 1982, 1983, 1990, 1992, 1994).

In this study, we aim to elucidate the Clastogenicity of 8 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) which are briefly summarized as follows.

### 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) and propylene glycol (PG) was obtained from Junsei Chemicals (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.

### *In vivo* bone marrow micronucleus assay after intraperitoneal administration in mice

The test article was applied intraperitoneally 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 hour intraperitoneal 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) issues 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 become 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, $LD_{50}$ values and CAS number

**Table 1.** 50% lethal dose ($LD_{50}$) of 8 synthetic chemicals after intraperitoneal administration in mice

| Chemical name | CAS No. | Cat. No. | Manufactured by | $LD_{50}$ (m,g/kg) | Reference |
|---|---|---|---|---|---|
| 1. Phenylisocyanate | 103-71-9 | 18535-3 | A | 100 | Our Data |
| 2. m-Aminochlorobenzene | 108-42-9 | 802812-0250 | M | 200 | RTECS |
| 3. 2-(2-Ethoxyethoxy)ethanol | 111-90-9 | D1265 | S | 3,905 | RTECS |
| 4. 2-Chloro-4-nitroaniline | 121-87-9 | 10165-6 | A | 500 | RTECS |
| 5. N-Butylchloride | 1009-69-3 | | B | 6,000 | Our Data |
| 6. Neopentyl glycol | 126-30-7 | | K | 6,400 | CHRIS |
| 7. Dicyanodiamine | 464-58-5 | | E | >4,000 | CHRIS |
| 8. 3,4-Dichlorobenzotrifluoride | 328-84-7 | 23580-6 | A | 1,150 | HSDB |

A : Aldrich Chemical Co. Inc., WI., USA, B: Bayer AG, Leverkusen, Germany
M : Merck AG., Darmstadt, Germany, S : Sigma Chemical Co. Ltd., Seoul, Korea
K: Korea Chemical Co. Ltd., Seoul, Korea, E: Eyang Chemical Co. Ltd., Seoul, Korea
RTECS : Registry of Toxic Effects of Chemical Substances
CHRIS : Chemical Hazards Response Information System
HSDB : Hazardous Substances Data Bank

of test chemicals were listed in Table 1. Among the many synthetic chemicals used in chemical reaction processes in industry, for example, phenylisocyanate (CAS No. 103-71-9) used as reagent for identifying alcohols and amines, m-aminochlorobenzene (CAS No. 108-42-9) used as a chemical intermediate for the herbicide, azo dyes, pigments, insecticide and agricultural chemicals. 2-Chloro-4-nitroaniline (CAS No. 121-87-9) used as intermediate in manufacture of disperse dyes. Neopentyl glycol (CAS No. 126-30-7) used in the manufacture of plasticizers, polyesters, and as modifier of alkyl resins. 3, 4-Dichlorobenzotrifluoride (CAS No. 328-84-7) is intermediate for insecticides and herbicides. 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 and all chemicals administered intraperitoneally. The administration dose for each experiment was determined with half dose of $LD_{50}$ value as high dose. The $LD_{50}$ of phenylisocyanate (CAS No. 103-71-9) and N-butylchloride (CAS No. 1009-69-3) was determined as 100 and 6,000 mg/kg, respectively, by Lorke method in our laboratory (Table 1).

**Table 2.** Micronucleus data on the bone marrow of ICR male mice i.p. administered with 8 chemicals.

| Test chemicals (CAS No.) | Dose (mg/kg) | MNPCE %/PCE[b] (Mean±SD) | Ratio % of PCE/PCE+NCE[c] (Mean±SD) | $P$-value[a] |
|---|---|---|---|---|
| CMC[d] | – | 0.22±0.15 | 0.48±0.01 | – |
| Phenylisocyanate | 50 | 0.20±0.03 | 0.51±0.01 | >0.05 |
| (103-71-9) | 25 | 0.20±0.03 | 0.48±0.01 | >0.05 |
| | 12.5 | 0.17±0.03 | 0.49±0.02 | >0.05 |
| m-Aminochlorobenzene | 100 | 0.18±0.10 | 0.48±0.01 | >0.05 |
| (108-42-9) | 50 | 0.28±0.10 | 0.49±0.02 | >0.05 |
| | 25 | 0.20±0.09 | 0.48±0.01 | >0.05 |
| 2-(2-Ethoxyethoxy)ethanol | 1,952 | 0.20±0.13 | 0.47±0.02 | >0.05 |
| (111-90-9) | 976 | 0.18±0.10 | 0.45±0.02 | >0.05 |
| | 488 | 0.13±0.10 | 0.47±0.02 | >0.05 |
| 2-Chloro-4-nitroaniline | 250 | 0.28±0.17 | 0.48±0.02 | >0.05 |
| (121-87-9) | 125 | 0.18±0.07 | 0.50±0.01 | >0.05 |
| | 62.5 | 0.25±0.05 | 0.48±0.02 | >0.05 |
| N-Butylchloride | 3,000 | 0.18±0.15 | 0.50±0.01 | >0.05 |
| (1009-69-3) | 1,500 | 0.18±0.12 | 0.49±0.01 | >0.05 |
| | 750 | 0.23±0.15 | 0.47±0.02 | >0.05 |
| Neopentyl glycol | 3,200 | 0.13±0.10 | 0.50±0.01 | >0.05 |
| (126-30-7) | 1,600 | 0.20±0.13 | 0.49±0.01 | >0.05 |
| | 800 | 0.25±0.16 | 0.48±0.01 | >0.05 |
| Dicyanodiamine | 2,000 | 0.22±0.17 | 0.49±0.01 | >0.05 |
| (464-58-5) | 1,000 | 0.25±0.16 | 0.49±0.01 | >0.05 |
| | 500 | 0.17±0.08 | 0.50±0.01 | >0.05 |
| 3,4-Dichlorobenzotrifluoride | 575 | 0.23±0.10 | 0.49±0.01 | >0.05 |
| (328-84-7) | 288 | 0.18±0.08 | 0.49±0.01 | >0.05 |
| | 144 | 0.20±0.09 | 0.49±0.01 | >0.05 |
| MMC[e] | 2 | 2.38±0.87 | 0.49±0.01 | 0.0000 |

[a]Pairwise comparison to corresponding control, significant at $P<0.05$
[b]MNPCE %/PCE: Percentage of Micronucleuated polychromatic erythrocytes/1,000 Polychromatic erythrocytes
[c]PCE/PCE+NCE: Polychromatic erythrocytes/1,000 erythrocytes
[d]CMC: 0.5% Carboxymethyl cellulose sodium Salt [e]MMC: Mitomycin C

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 CMC control. The constant range of ratio percentage of polychromatic erythrocytes/1,000 erythrocytes was also observed in all experiments as summarized in Table 2. The chemicals with relatively high $LD_{50}$ value such as phenylisocyanate (CAS No. 103-71-9), m-aminochlorobenzene and 2-chloro-4-nitroaniline revealed no significant induction of micronucleated polychromatic erythrocytes in mice. In this experiment, 8 synthetic chemicals well used in industry have revealed no significant micronucleus induction of clastogenicity in mice (Table 2).

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) to distinguish immature erythrocytes in the peripheral bloods was introduced instead of mouse bone marrow micronucleus assay. 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 (Tanisho *et al.*, 1998; Hayashi *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 was 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.*, 1999).

## ABSTRACT

To validate and to estimate the chemical hazard play a very important role to environment and human health. The detection of many synthetic chemicals used in industry that may pose a genetic hazard in our environment is of great concern at present. Since these substances are not limited to the original products, and enter the environment, they have become widespread environmental pollutants, thus leading to a variety of chemicals that possibly threaten the public health.

In this resepct, the clastogenicity of 8 synthetic chemicals was evaluated with bone marrow micronucleus assay in mice. The positive control, mitomycin C (2 mg/kg, i.p.) revealed significant induction ratio of percentage of micronucleated polychromatic erythrocytes/1,000 polychromatic erythrocytes compared to carboxymethylcellulose control. The chemicals with relatively high $LD_{50}$ value such as phenylisocyanate (CAS No. 103-71-9), m-aminochlorobenzene (CAS No. 108-42-9) and 2-chloro-4-nitroaniline (CAS No. 121-87-9) revealed no significant induction of micronucleated polychromatic erythrocytes in mice. From this results, 8 synthetic chemicals widely used in industry have revealed no significant micronucleus induction of clastogenicity in mice in this experiment.

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