Environmental Mutagens & Carcinogens 22-4 : 215-222 (2002)

Evaluation of the genetic toxicity of synthetic chemicals (V) – *in vitro* Chromosomal Aberration Assay with 17 chemicals in Chinese Hamster Lung Cells –

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(Received October 2, 2002 / Accepted October 20, 2002)

***ABSTRACT*: 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 respect, to regulate and to evaluate the chemical hazard will be important to environment and human health. The clastogenicity of 17 synthetic chemicals was evaluated in Chinese hamster lung fibroblast cells *in vitro*. Two most cytotoxic chemicals, dodecyl methacrylate (CAS No. 142-90-5) and 2-ethylhexyl methacrylate (CAS No. 688-84-6), among 17 chemicals tested revealed no clastogenicity in the range of 0.0165-0.066 μg/ml and 0.006-0.024 μg/ml both in the presence and absence of metabolic activation system, respectively. All 17 chemicals revealed no significant induction of chromosomal aberration both in the presence and absence of metabolic activation system in this assay. From the results of chromosomal aberration assay with 17 synthetic chemicals in Chinese hamster lung cells *in vitro*, we did not observed positive clastogenic results in this study.**

Keywords : **Genotoxicity, Clastogenicity, *in vitro* Chromosome Aberration, Chinese Hamster Lung Fibroblast**

Introduction

The establishment of toxicity and detection of synthetic chemicals that may pose a genetic hazard in our environment is subjects of great concern at present (WHO, 1971) because there are many synthetic chemicals used in chemical reaction processes in industry.

Several assay systems having rapidity and reliability have been introduced for this purpose, 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), micronucleus assay with rodents (Hayashi *et al.*, 1992; Schmid, 1975). These assay systems are now well used to evaluate the genotoxicity of chemicals and also frequently adopted as methods for an index of genotoxicity worldwide. Furthermore, it was well applied as a screening probe for the detection of possible carcinogenic substances in our environment. Since the tens of thousands of man-made chemicals that have been introduced into the environment in the last few decades must also be tested for their damaging effect on DNA, the agents that cause this damage must be identified. Generally, the carcinogenicity of chemicals is one of the potential toxicity that may consider for the human health. As one of the mechanisms of carcinogenicity, induction of DNA damage was ascertained by several genotoxicity assays.

Despite the many toxicological researches on synthetic chemicals, there are few reports on the genotoxicity of some chemicals especially well used in chemical reaction processes in industry. In this respect, administrative authority has great concern to regulate and to evaluate the chemical hazards, and conducted the toxicity evaluations of synthetic chemicals. Our laboratory had also been involved in toxicity evaluation, especially in genotoxicity (Ryu *et al.*, 1993a, 1994, 1996a,b, 1997, 1998a,b,c,d, 1999a,b, 2000, 2001a,b,c,d, 2002a, b; Kim *et al.*, 2001).

In this study, we aim to elucidate the clastogenicity of 17 synthetic chemicals used in chemical process with CHL cells *in vitro*.

Materials and Methods

The experiment was performed as described by OECD

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(1993) and Ishidate and Odashima (1977) with some minor modifications (Ryu *et al.*, 1993a, 1994, 1996a,b, 1998a,b, 2001b, 2002b) which are briefly summarized as follows.

Cell Culture

A clonal sub-line of a chinese hamster lung (CHL) fibroblast cells was obtained from the National Institute of Health Sciences, Tokyo, Japan. The karyotype of CHL cells consisted of 25 chromosomes. The cells had been maintained by 3-4 day passages and grown in a monolayer with Eagles minimum essential medium (EMEM, Gibco, 410-1100EA) supplemented with 10% fetal bovine serum (FBS, Gibco, 26140-020). These cells were maintained at 37°C in 5% CO_2 atmosphere.

Reagents

Trypsin-EDTA and colcemid were the products of Gibco BRL Life Tech. Inc. (Gaithersburg, USA). The test chemicals were kindly donated and purchased from several companies as indicated in Table 2. The test compounds were dissolved in dimethylsulfoxide (DMSO). The preparation of rat liver S-9 fraction for metabolic activation system was previously reported (Ames et al., 1973; Maron and Ames, 1983). The S-9 fraction prepared was stored immediately at –80°C before use.

Determination of the 50% growth inhibition concentration

Test article dose levels were determined prior to the main study in a dose range-finding study performed in the absence of a rat liver S-9 activation system. For the growth inhibition assay, CHL cells were seeded at the density of 5×10^4 cells/ml into 96 well plates. Twenty-four hours after seeding, several different doses of sample were separately added and incubated for 24 hours. And then the 50% inhibition concentration (IC_{50}) values were calculated by MTT assay (Mosmann, 1983).

Chromosome aberration assay

For the aberration assay, three different doses, including the IC_{50} value as a maximum dose, were prepared and separately added to 3-day-old cultures (approximately 10^5 cells/60 mm dish). In the absence of metabolic activation, cultures were treated for 24 hours with the test article, while in the presence of metabolic activation, cells were treated for 6 hours because of toxicity of S-9 and then maintained for 18 hours in the fresh medium to adjust a time equivalent to about 1.5 normal cell cycle lengths. Treatment was followed by addition of medium containing colcemid at a concentration of 0.2 μg/ml. After 2 hr further incubation in the presence of colcemid, metaphase cells were harvested by centrifugation and trypsinization. The cells were swollen with hypotonic (0.075 M) KCl solution for 20 min at 37°C, and washed three times in ice-cold fixative (methanol : glacial acetic acid = 3 : 1). After centrifugation, the fixative was removed, and cell pellet suspensions were prepared by pipetting gently. A few drop of cell pellet suspension were dropped onto precleaned glass microscope slides, and air dried. Slides were stained with 5% Giemsa buffered solution at pH 6.8 for scoring of chromosome aberrations. The number of cells with chromosomal aberrations was recorded on 200 well-spread metaphases at the magnification of 1,000 with Axioscope microscope (Karl Zeiss, FRG). The classification of aberration types referred to JEMS-MMS (1988). Breaks less than the width of a chromatid were designated as gaps in our criteria, and not included as chromosomal aberration. The incidence of polyploid and endoreduplicated cells was also recorded when these events were observed. Solvent-treated cells served as controls in this experiment.

Evaluation

CHL cells usually have less than 3.0% cells with spontaneous chromosome aberrations. Aberration frequencies, defined as aberrations observed divided by number of cells counted, were analyzed using Fishers exact test (Altman, 1993) with Dunnetts adjustment and compared with results from the solvent controls. Therefore, data from count up well-spread 200 metaphase cells were expressed as percentages, and then dose-dependent responses and the statistical significance in *p*-value will be considered as positive results in our judgement.

Results and Discussion

It is well known that carcinogenicity of synthetic chemicals is the most serious problem in human health hazard. As one of the mechanisms of carcinogenicity, it has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of 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). Moreover, mutagens

and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement (Zimmermann, 1971) which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of cellular cancer genes (Radman *et al.*, 1982).

Several short term methods have been developed (Ames *et al.*, 1973; Maron and Ames, 1983; Mersch-Sundermann *et al.*, 1991) for predicting the carcinogenicity of chemicals and also been introduced to the evaluation of genotoxicity (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979; Radman *et al.*, 1982; Hayashi *et al.*, 1982, 1990, 1992; Ryu *et al.*, 1993a, 1994, 1996a,b, 1997, 1998a,b,c,d, 1999a,b, 2000, 2001a,b,c,d, 2002a,b) and of antimutagenicity (Sato *et al.*, 1991; Ryu *et al.*, 1993b, 2001c). Cytogenetic studies on mammalian cells *in vivo* (Schmid, 1975; Hayashi *et al.*, 1982, 1990, 1992, 1994; Heo *et al.*, 1997) as well as in vitro (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979) have also been widely used as a screening method for DNA-attacking substances.

The chemical name and CAS No. of test chemicals were listed in Table 1 and their uses in industry are diverse. Among the chemicals subjected in this experiment, for example, methyl carbitol i.e. 2-(2-methoxyethoxy ethanol) (CAS No. 111-77-3) and 2-[2-(2-methoxyethoxy)ethoxy] ethanol (CAS No. 112-35-6) are well used in the same way as 2-ethoxyethanol where a solevnt with a higher boiling point required. Triphenyl phosphine (CAS No. 603-35-0) is important in organic synthesis as polymerization initiator, and tritolyl phosphate (CAS No. 1330-78-5) used as plasticizer, flame-retardant and additive to extreme pressure lubricants. Since these substances are not limited to the original products, and they may enter the environment and have become widespread environmental pollutants, thus leading to a variety of chemicals that possibly threaten the public health. Nevertheless of the diverse uses of these chemicals in industry, however, there has been no attention to evaluate the toxicity of some chemicals especially used in chemical industry. The detection and the regulation of man-made synthetic chemicals are subjects of great concern because of its close correlation between environmental contamination and human health.

We used CHL cells in this experiment because it was reported no diffrences of sensitivity between CHL and CHO (Chinese hamster ovary) cells in *in vitro* chromosome aberration study (Galloway *et al.*, 1997). It was also reported that extended harvest times are not necessary for the detection of *in vitro* clastogens in regulatory cytogenetic studies except it might help to resolve an equivocal result by Henderson *et al.* (1996). The IC_{50} values of cell growth of test articles in CHL cells are obtained in the absence of metabolic activation system as shown in Table 1.

Two most cytotoxic chemicals, dodecyl methacrylate (CAS No. 142-90-5) and 2-ethylhexyl methacrylate (CAS No. 688-84-6), among 17 chemicals tested revealed no clastogenicity in the range of 0.0165-0.066 μg/ml and 0.006-0.024 μg/ml both in the presence and absence of metabolic activation system, respectively. The concentration used and detailed data of chromosome aberration of 17 chemicals are summarized in Table 2. The DMSO negative control is revealed only 1.5% and 1.2% spontaneous aberrations in the absence and presence of metabolic

Table 1. 50% Cell Growth Inhibition concentration (IC_{50}) of 17 synthetic chemicals in Chinese hamster lung cells

Chemical Name	CAS No.	Cat. No.	IC_{50} (μg/ml)
1. 2-(2-Methoxyethoxyethanol)	111-77-3	10990-8	1,200.0
2. 2-[2-(2-Methoxyethoxy)ethoxy]ethanol	112-35-6	T0709	1,600.0
3. 2-[2-(2-Ethoxyethoxy)ethoxy]ethanol	112-50-5	E0167	1,780.0
4. Octadecyl isocyanate	112-96-9	0-108-7	73.8
5. Trimethyl phosphite	121-45-9	T4888	124.0
6. 2-(2-Butoxyethoxy)ethanol acetate	124-17-4	30706-8	1,020.0
7. 2,6-Di-tert-butylphenol	128-39-2	D4840-0	1.03
8. Dodecyl methacrylate	142-90-5	29181-1	0.066
9. Bis(2-ethylhexyl)phosphate	298-07-7	23782-5	0.13
10. 2,4-Dichloroaniline	554-00-7	11215-1	20.25
11. Triphenyl phosphine	603-35-0	T1136	2.62
12. Dimethyldisulfide	624-92-0	D8501	9.4
13. 2-Ethylhexyl methacrylate	688-84-6	M0591	0.024
14. Tritolyl phosphate	1330-78-5	26891-7	368.4
15. m-Phenylene bis-methylamine	1477-55-0	D0127	136.2
16. Trixylenyl phosphate	25155-23-1	9792	4,100.0
17. Diphenyl tolyl phosphate	26444-49-5	P0259	3,400.0

Table 2. Chromosome aberration assay of 17 chemicals in Chinese hamster lung cells

Test chemicals (CAS No.)	Manu-factured by	Concen-tration (μg/ml)	without (−) or with (+) S9 mix	Aberration Frequency (%)				Total aberration (%)	Extra aberrations (%)			
				Chromatid		Chromosome						
				Br	Ex	Br	Ex		ctg	csg	poly	endo
DMSO			−	1.2±0.5	0	0.3±0.1	0	1.5±0.5[a)]	0.7	0	0.1	0
			+	0.8±0.3	0	0.4±0.2	0	1.2±0.4[a)]	0.5	0	0	0
2-(2-methoxyethoxy ethanol) (111-77-3)	A	1,200	−	0	0	0	0	0	3	0	0	0
		600	−	0	0	0	0	0	3	1	0	0
		300	−	0	0	0	0	0	2	0	1	0
		1,200	+	0	0	0	0	0	2	0	0	0
		600	+	0	0	0	0	0	1	0	0	0
		300	+	0	0	0	0	0	1	0	0	0
2-[2-(2-methoxyethoxy) ethoxy] ethanol (112-35-6)	T	1,600	−	1	0	1	0	2	1	0	0	0
		800	−	0	0	0	0	0	1	0	1	0
		400	−	0	0	0	0	0	0	0	0	0
		1,600	+	0	0	0	0	0	1	0	0	1
		800	+	0	0	0	1	1	0	0	0	0
		400	+	0	0	0	0	0	2	0	1	0
2-[2-(2-ethoxyethoxy) ethoxy] ethanol (112-50-5)	T	1,780	−	1	0	0	1	2	2	0	0	0
		890	−	0	0	0	0	0	4	0	0	0
		445	−	1	0	0	0	1	2	0	0	0
		1,780	+	0	0	0	0	0	1	0	0	0
		890	+	0	0	0	1	1	0	0	1	0
		445	+	1	0	0	0	1	1	0	0	0
Octadecyl isocyanate (112-96-9)	A	73.8	−	1	0	0	0	1	2	1	1	0
		36.9	−	1	0	0	0	1	1	0	0	0
		18.45	−	0	0	0	1	1	2	0	0	0
		73.8	+	0	0	0	0	0	1	0	0	0
		36.9	+	0	0	0	1	1	1	0	0	0
		18.45	+	1	0	0	0	1	2	0	1	0
Trimethyl phosphite (121-45-9)	S	124	−	0	1	1	0	2	1	0	0	0
		62	−	0	0	0	1	1	1	0	0	1
		31	−	0	0	0	0	0	1	0	0	0
		124	+	0	0	0	0	0	2	2	0	0
		62	+	1	0	1	0	2	0	0	0	0
		31	+	0	0	0	0	0	2	0	0	0
2-(2-butoxyethoxy) ethanol acetate (124-17-4)	A	1,020	−	1	0	0	0	1	2	0	1	0
		510	−	1	0	0	0	1	1	0	0	0
		255	−	0	1	0	0	1	0	0	0	0
		1,020	+	0	0	0	1	1	1	0	0	0
		510	+	1	0	0	1	2	2	0	1	0
		255	+	0	0	0	0	0	1	0	0	0
2,6-di-tert-butylphenol (128-39-2)	A	1.03	−	0	0	0	0	0	0	0	0	0
		0.515	−	0	0	0	0	0	0	0	0	0
		0.258	−	0	0	0	0	0	1	0	0	0
		1.03	+	0	0	0	0	0	2	0	1	0
		0.515	+	0	0	0	1	1	1	0	0	0
		0.258	+	0	0	1	0	1	1	0	0	0
Dodecyl methacrylate (142-90-5)	A	0.066	−	0	0	0	0	0	2	0	0	0
		0.033	−	0	0	0	0	0	1	0	0	0
		0.0165	−	1	0	0	1	2	1	0	0	0
		0.066	+	0	0	0	0	0	3	0	0	0
		0.033	+	0	0	0	0	0	1	0	1	0
		0.0165	+	0	0	0	0	0	1	0	0	0

Table 2.

Test chemicals (CAS No.)	Manufactured by	Concentration (μg/ml)	without (–) or with (+) S9 mix	Aberration Frequency (%)				Total aberration (%)	Extra aberrations (%)			
				Chromatid		Chromosome						
				Br	Ex	Br	Ex		ctg	csg	poly	endo
Bis(2-ethylhexyl) phosphate (298-07-7)	A	0.13	–	0	0	1	0	1	2	0	0	0
		0.065	–	1	0	1	1	3	0	0	0	0
		0.0325	–	0	0	0	0	0	1	0	0	0
		0.13	+	0	0	0	1	1	2	0	0	0
		0.065	+	0	0	0	0	0	1	0	0	0
		0.0325	+	0	0	0	0	0	2	0	2	0
2,4-Dichloroaniline (554-00-7)	A	20.25	–	0	0	0	0	0	1	0	0	0
		10.13	–	0	1	0	0	1	1	0	1	0
		5.06	–	0	1	0	0	1	0	0	0	0
		20.25	+	0	1	0	0	1	2	0	0	0
		10.13	+	0	1	0	0	1	1	0	0	0
		5.06	+	0	1	0	0	1	1	1	0	1
Triphenyl phosphine (603-35-0)	S	2.62	–	0	0	0	1	1	2	0	0	0
		1.31	–	0	0	0	0	0	1	0	0	0
		0.66	–	1	0	0	0	1	1	0	2	0
		2.62	+	2	0	0	0	2	2	0	0	0
		1.31	+	0	0	0	1	1	1	0	0	0
		0.66	+	0	0	0	0	0	2	0	0	0
Dimethyldisulfide (624-92-0)	S	9.4	–	0	0	0	0	0	4	0	0	0
		4.7	–	0	0	1	0	1	1	0	0	0
		2.35	–	0	0	0	0	0	1	0	0	0
		9.4	+	1	1	0	0	2	2	0	0	0
		4.7	+	1	0	0	0	1	2	0	0	0
		2.35	+	0	0	0	0	0	1	0	0	0
2-Ethylhexyl methacrylate (688-84-6)	T	0.024	–	0	0	1	0	1	2	0	0	0
		0.012	–	0	0	0	0	0	1	0	0	0
		0.006	–	0	1	0	0	1	0	0	0	0
		0.024	+	1	0	0	0	1	1	0	0	0
		0.012	+	1	0	0	0	1	2	0	0	0
		0.006	+	0	0	0	0	0	2	0	0	0
Tritolyl phosphate (1330-78-5)	A	368.4	–	1	0	0	0	1	2	0	0	0
		184.2	–	0	0	0	0	0	1	0	0	0
		92.1	–	0	0	0	0	0	2	0	0	0
		368.4	+	1	0	0	0	1	1	0	0	1
		184.2	+	1	0	0	0	1	0	0	0	0
		92.1	+	0	0	0	0	0	2	0	0	0
m-phenylene bis-methyl amine (1477-55-0)	T	136.2	–	0	0	0	0	0	3	0	0	0
		68.1	–	1	0	0	0	1	2	0	0	0
		34.1	–	0	0	0	0	0	2	0	0	0
		136.2	+	0	0	1	0	1	1	0	0	0
		68.1	+	0	0	0	0	0	2	0	0	0
		34.1	+	0	0	0	0	0	1	0	0	0
Trixylenyl phosphate (25155-23-1)	M	4,100	–	0	0	0	0	0	2	0	0	0
		2,050	–	0	0	0	0	0	1	0	1	0
		1,025	–	0	0	0	0	0	1	0	0	0
		4,100	+	0	0	0	0	0	1	0	0	0
		2,050	+	0	0	0	0	0	2	0	0	0
		1,025	+	0	0	0	0	0	1	0	0	0

Table 2.

Test chemicals (CAS No.)	Manu-factured by	Concen-tration (μg/ml)	without (−) or with (+) S9 mix	Aberration Frequency (%)				Total aberration (%)	Extra aberrations (%)			
				Chromatid		Chromosome						
				Br	Ex	Br	Ex		ctg	csg	poly	endo
Diphenyl tolyl phosphate (26444-49-5)	T	3,400	−	0	0	0	0	0	1	0	0	0
		1,700	−	0	0	0	0	0	1	0	0	0
		850	−	0	0	0	0	0	1	0	0	0
		3,400	+	0	0	0	0	0	1	0	0	0
		1,700	+	0	0	0	0	0	1	0	0	0
		850	+	1	0	0	0	1	1	0	1	0
MMC	S	0.1	−	13.3±4.6	16.4±3.4	8.2±3.1	2.0±0.5	32.9±5.5[a)]	5.2	1.5	0.2	0
B(a)P	S	200	+	9.2±4.1	5.5±3.8	5.4±3.0	1.5±1.0	18.9±5.5[a)]	4.6	0.5	0	0

*significant at p<0.05.
Br: Breakage Ex: Exchange, ctg: chromatid gap, csg: chromosome gap, poly: polyploid, endo: endoreduplicate.
DMSO: dimethlysulfoxide, MMC: mitomycin C, B(a)P: benzo(a)pyrene.
[a)]The values of solvent and positive controls are expressed as mean ± S.D.
A: Aldrich Chemical Co. Inc., WI., USA, M: Merck AG., Darmstadt, Germany.
S: Sigma Chemical Co. Ltd., Seoul, Korea. T: Tokyo Casei Inc., Japan.

activation system in 200 metaphase of CHL cells, respectively. However, the positive controls, benzo (a)pyrene (200 μg/ml) as an indirect mutagen that require metabolic activation and mitomycin C (0.1 μg/ml) as a direct-acting mutagen, induced remarkable chromosome aberrations (18.9-32.9%) in CHL cells as shown in Table 2.

From the results of chromosomal aberration assay with 17 synthetic chemicals in Chinese hamster lung cells, no remarkable positive clastogenicity was not observed in this study.

Recently, several next generation battery of genotoxicity for the detection of genetic damages *in vitro* and *in vivo* were introduced according to the rapid progress in toxicology combined with cellular and molecular biology. Among these methods, the single cell gel electrophoresis (comet assay) which can be detected DNA damages in cell level (Singh *et al.*, 1994; Ryu *et al.*, 1997, 2001a,d; Tice *et al.*, 2000), mouse lymphoma thymidine kinase gene assay (Clive *et al.*, 1983; Sawyer *et al.*, 1985; Ryu *et al.*, 1999a), FISH (fluorescence in situ hybridization) (Hayashi *et al.*, 1994), PRINS (primed *in situ* hybridization) (Abbo *et al.*, 1993) and transgenic animal and cell line model as a parameter of *lac I* (Big Blue) (Kohler *et al.*, 1991; Ryu *et al.*, 1998c,d, 1999b, 2000, 2002a) or *lac Z* (Muta Mouse) (Suzuki *et al.*, 1993) gene mutation are newly introduced based on cellular and molecular toxicological approaches. Also, *in vivo* supravital micronucleus assay with peripheral reticulocytes by using acridine orange fluorescent staining (Hayashi *et al.*, 1990, 1992; Ryu *et al.*, 1998b) was introduced instead of mouse bone marrow micronucleus assay. Our laboratory is now under progress these assays to evaluate and to elucidate the mechanism of genetic toxicity and/or carcinogenesis, and will be presented in near future.

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