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Comparison of L5178Y tk$^{+/-}$ Mouse Lymphoma Assay and *In vitro* Chromosome Aberration Test

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ABSTRACT *: The mouse lymphoma assay (MLA) has been recently validated as a sensitive and specific test system to determine the genotoxic potential for a chemical. The objective of this study is to evaluate the utility of MLA for detecting mutagens. Especially, to compare MLA with the in vitro chromosomal aberration test (CA), we performed MLA using the microwell method with three chemicals (hydroxyurea, theophylline and amino acid copper complex), which were reportedly positive in the CA. In cells treated with hydroxyurea, anti-neoplastic agent that blocks DNA replication, evidence of a positive response was obtained without S9 mix for 4 h and 24 h. In addition, analysis of colony size distribution at concentration that gave an elevated mutant fraction showed that hydroxyurea induced a high proportion of small type colonies, indicating that hydroxyurea-induced mutation is associated with large chromosomal deletions. Conversely, negative MLA result was obtained for theophylline, which was used as central nervous system stimulator. Although theophylline increased the mutant frequency at concentration of 1250 μg/ml with S9 mix for 4 h, a concentration-related increase in mutant frequency was not observed. The MLA result of amino acid copper complex was considered equivocal because the positive result was obtained at concentrations showing 10% or less RS or RTG. Thus, among 3 CA-positive chemicals, positive MLA result was obtained for one. The other two chemicals were negative and equivocal. However. MLA, which evaluates mutagenic potential of chemicals through colony formation by cell growth, may provide a higher predictivity of carcinogenesis than CA.*

Key Words *: Mouse lymphoma assay, Hydroxyurea, Theophylline, Genotoxicity*

I. INTRODUCTION

According to biological system used and endpoints detected, many genotoxicity tests have been developed and used. At present, the guidelines of most country recommend a battery of tests because no single test is capable of detecting all genotoxic chemicals. Generally, three battery consists of a bacterial reverse mutation assay (Ames test), an *in vitro* chromosome aberration assay (CA) and an *in vivo* rodent micronucleus assay (MN). Especially, US Environmental Protection Agency (EPA) recommends three test battery including L5178Y tk$^{+/-}$ mouse lymphoma assay (MLA) instead of CA (Dearfield *et al.*, 1991). Recently, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) states that CA and MLA test are considered interchangeable in the standard battery tests, providing appropriate protocols are used (ICH, 1997).

MLA was originally developed by Clive *et al.* (1972) using soft agar medium to clone and enumerate mutants. Subsequently, the assay using a microwell protocol was established for cloning and mutant detection (Cole *et al.*, 1986). At present, ICH suggested a preference for microwell over soft agar, although the International Workshop on Genotoxicity Test Procedures (IWGTP) workgroup concluded that there was insufficient data to recommend one version

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The List of Abbreviation : MLA, mouse lymphoma assay; CA, chromosomal aberration test; RS, relative survival; RSG, relative suspension growth; RTG, relative total growth; PE, plating efficiency; TFT, trifluorothymidine; TK, thymidine kinase; NQO, 4-nitroquinoline-1-oxide; BP, benzo(a)pyrene

over the other and that both versions of the assay were equally acceptable (Moore *et al.*, 2000). MLA is the short-term test for measuring the induction of thymidine kinase (tk)-deficient mutants (Clive *et al.*, 1972). In cells, DNA can be made either de novo or through a salvage pathway that uses molecular skeletons already available. The target gene, thymidine kinase, which is a dispensable gene, is involved in a salvage pathway for thymidine derived from DNA metabolism (Cole and Arlett, 1984). Mutants can be selected and mutant frequencies derived by including a thymidine analogue, trifluorothymidine (TFT) in the culture medium of cells after exposure to test chemicals. Normal cells with intact tk incorporate the analogue forming TFT-monophosphate and die (Clive *et al.*, 1987). In contrast, mutant cells, which lack tk activity, survive, form colonies and are quantified.

With the recent development of MLA methods (Amacher *et al.*, 1980), many laboratories compared MLA with other testing methods, especially *in vitro* chromosome aberration assay. Snyder *et al.* (2001) reported that *in vitro* CA and MLA exhibited higher frequencies of positive results compared with *in vivo* MN test, and bacterial reverse mutation assay showed the strongest association with rodent carcinogenicity (Snyder *et al.*, 2001). On the other hand, the reports from the Ministry of Health and Welfare of Japan and the Japanese Pharmaceutical Manufacturer's Association concluded that MLA was not as sensitive as the CA (Honma et al., 1999a). However, US regulatory authorities advocate inclusion of the MLA rather than the CA into the standard genotoxic test battery, because MLA can detect a wide range of genetic damage, including point mutations in addition to gross structural and numerical changes ay the chromosomal level (Combes *et al.*, 1995). In MLA, the different degrees of genetic damage results in mutant colonies, which can be classified by colony size as large and small. In fact, small colonies with slow growth kinetics may result from events that affect not only the tk locus but also additional genes whose inactivation cause the cells to be slow growing, while large colonies result from mutational events affecting the expression of only the tk locus (Moore *et al.*, 1985; Applegate *et al.*, 1990).

In this study, we compared MLA with CA for three test chemicals, hydroxyurea, theophylline and amino acid copper complex, which have been previously identified as positive in CA. Among three CA-positive chemicals, positive MLA result was obtained for hydroxyurea. Theophylline and amino acid copper complex were negative and equivocal, respectively.

II. MATERIALS AND METHODS

1. Test chemicals

In this study, three CA-positive chemicals were used for MLA. Amino acid copper complex was from Yosu National University (Yosu, Korea). Hydroxyurea and theophylline were purchased from Sigma (St. Louis, MO). Amino acid copper complex, which was blue-colored liquid, was diluted with culture media for each experiment. Hydroxyurea and theophylline were also dissolved in culture media.

2. Materials

Most chemicals including positive controls such as 4-nitroquinoline 1-oxide (NQO) and benzo(a)pyrene were obtained from Sigma. RPMI 1640 medium, horse serum, penicillin, and streptomycin were purchased from GIBCO BRL (Grand Island, NY). S9, which was prepared from male Sprague-Dawley rats induced with Aroclor 1254, was from Molecular Toxicology Inc. (Boone, NC) and cofactor for S9 mix was from Wako Pure Chem. Ind., Ltd. (Japan). S9 mix was used at concentration of 3% in the final culture medium. Both of positive controls were dissolved in dimethylsulfoxide (DMSO). NQO stock concentration was 10 μg/ml yielding final concentration of 0.1 μg/ml in the cultures for 4 h treatments. Final concentration of 0.04 μg/ml NQO was used for 24 h treatment. Benzo(a)pyrene, which was a typical positive control chemicals in the presence of S9, was used at concentration of 3 μg/ml.

3. Cell line

MLA was performed using L5178Y $tk^{+/-}$ mouse lymphoma cells, which were established by Fischer (1958), as described by Clements (2000). L5178Y $tk^{+/-}$ mouse lymphoma cells were obtained from LGCI Ltd (Daejeon, Korea). The cells were cultured in RPMI

1640 medium supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 10% heat-inactivated horse serum. Subculturing was conducted every 2~3 days so as not to exceed 2×10^6 cells/ml.

4. Cleansing of cultures

The spontaneous mutant frequency increases with subculturing. Therefore, to prevent high background arising from spontaneous mutations, cleansing of the cultures should be done just prior to preparing cells for a treatment (Nestman *et al.*, 1991). The cells lacking tk gene can be killed by culturing cells RPMI 1640 medium containing THMG (3 μg/ml thymidine, 5 μg/ml hypoxanthine, 0.1 μg/ml methotrexate, and 7.5 μg/ml glycine). After 24 h, cells were resuspended in THG medium without methotrexate for 3 days. Thymidine, hypoxanthine and glycine force the cells to be dependent on TK salvage pathway of thymidine monophosphate (TMP) synthesis while methotrexate blocks the de novo synthetic pathway, confining DNA synthesis to the salvage pathway.

5. Treatment of cell cultures

For 4 h treatment, cell concentrations were adjusted to 1.2×10^6 cells/ml in RPMI 1640 medium containing 10% horse serum ($R_{10}P$). One ml of cells were dispensed to 15 ml tubes containing 0.78 ml (or 0.6 ml) RPMI 1640 buffer (R_0P). Freshly prepared S9 mix or R_0P (0.2 ml) was added to each tubes followed by 20 μl (or 0.2 ml) of test chemicals. The final reaction mixture in all cultures contained 2 ml of cells, at cell concentration of 6×10^5 cells/ml in R_5P medium. After a 4 h rocking at 37°C, the cells were centrifuged at 200 ×g for 10 min and resuspended in $R_{10}P$ to a cell concentration of 3×10^5 cells/ml. For 24-h treatment, cells were incubated with test chemicals at concentration of 3×10^5 cells/ml in 4 ml of $R_{10}P$ in 60 mm petri dish with rocking. After treatment with test chemicals, cells were transferred to T-75 flasks for growth through the expression period or diluted to be plated for survival assay. During expression period, cell concentrations were determined on Day 1, and each cultures adjusted to 3×10^5 cells/ml. After 48 h incubation, cells were used to estimate the viability and mutant selection assay.

6. Plating for survival and viability

After treatment of test chemicals (for survival assay) and at the end of the expression period of 2 days (for viability assay), the cells were centrifuged at 200 ×g for 10 min and resuspended in $R_{20}P$ cloning medium containing 20% horse serum, 1.9 mM sodium pyruvate and 2.5 μg/ml amphotericin B to a cell concentration of 8 cells/ml. 96 well microtiter plates were filled with 0.2 ml cell culture per well (1.6 cells/well). The plates were incubated at 37°C in an atmosphere of 5% CO_2/95% air for 9 days. The number of empty wells in each plate was identified by eye and counted.

7. Plating for 5-trifluorothymidine (TFT) resistance

At the end of the expression period of 2 days, cell concentrations were adjusted to give 1×10^4 cells/ml in $R_{20}P$ cloning medium containing 20% horse serum, 1.9 mM sodium pyruvate and 2.5 μg/ml amphotericin B. Trifluorothymidine (TFT) stock solution was added to these suspensions to give a final concentration of 3 μg/ml. 96 well microtiter plates were filled with 0.2 ml cell culture per well (2000 cells/well). The plates were incubated at 37°C in an atmosphere of 5% CO_2/95% air for 12 days. The number of empty wells in each plate was identified by eye and counted.

8. Data analysis

1) Assessment of survival or viability

The percentage relative survival (%RS) in each culture can be determined by comparison plating efficiencies in test and control cultures.

PE (plating efficiency) = P/No. of cells plated per well

① P = -ln(empty well/total well);

② An average of 1.6 cells per well were plated on survival and viability plates

Therefore, %RS (% relative survival)

$= [PE_{(test)}/PE_{(control)}]\times100$

In addition, relative total growth (RTG) was also calculated as follows. This value includes initial cell loss to toxicity and subsequent recovery during the

expression period.

RSG (relative suspension growth) = $SG_{(test)}/SG_{(control)}$,
SG = [Day 1 cell conc./Day 0 cell conc.]
×[Day 2 cell conc./Day 1 adjusted cell conc.]
Day 2 relative PE = Day 2 $PE_{(test)}$/Day 2 $PE_{(control)}$

Therefore, %RTG = RSG×Day 2 relative PE×100

2) Assessment of mutation frequency

The number of empty wells from viability assay and the number of empty wells from the TFT resistance assay were used to calculate the mutation frequency (MF).

$MF = [PE_{(mutant)}/PE_{(viable)}]\times 10^6$

① $PE_{(mutant)} = P_{(mutant)}/2\times 10^3$
(An average of 2000 cells per well were plated on TFT resistance plates)

② $PE_{(viable)} = P_{(viable)}/1.6$
Where, in each case, P = -ln(empty well/total well)

9. Colony sizing

The ratio of small type mutant colonies to large type mutant colonies should be expressed for negative and positive controls, and the highest positive concentration of test chemicals. This ratio provides additional information regarding the type of genetic damage being induced by positive test chemicals (Moore *et al.*, 1985; Applegate *et al.*, 1990). The small colonies are defined as less than a quarter of the diameter of the well. Additionally, small colonies have compact morphology. However, size is a key factor in defining small colonies and morphology is secondary. The size of colonies was identified by eye and counted.

10. Criteria for judgement

Positive responses are characterized by increment of mutant frequencies in treated groups compared to that in negative control and responses should be dose-dependent (Sofuni *et al.*, 1997; Moore *et al.*, 2000). The dose-dependancy is potentially analyzed by a linear-trend test. In particular, positive data obtained at concentrations showing less than 10% RS or RTG will normally be excluded from statistical evaluation.

III. RESULTS

1. Determination of dose range

A preliminary study was performed to determine the highest concentration, which should result in approximately 10~20% relative survival (%RS) or relative total growth (%RTG). The experimental data for the individual cultures conducted with and without S9 mix are included in Table 1. In 4 h treatment cytotoxicity range finder experiment, hydroxyurea showed 85~86% cytotoxicity at 1250 μg/ml and 156 μg/ml without and with S9 mix, respectively. In addition, %RTG was 23 at the concentration of 313 μg/ml when chemicals were treated for 24 h. Thus, concentration chosen for mutation assay ranged from 125 to 1000 μg/ml (for 4 h treatment), and 62.5 to 500 mg/ml (for 24 h continuous treatment) without

Table 1. Result of preliminary range-finding test

Conc.	4 hr (- S9)		4 hr (+ S9)		24 hr (- S9)	
	RSG[a]	RTG (%)[b]	RSG	RTG (%)	RSG	RTG (%)
Hydroxyurea (μg/ml)						
0	1.00	100	1.00	100	1.00	100
156	0.80	90	0.58	14	0.23	23
313	0.87	91	0.64	13	0.18	0
625	0.94	75	0.51	10	0.16	0
1250	0.53	15	0.43	8	0.16	0
2500	0.75	16	0.43	8	0.16	0
5000	0.68	14	0.38	6	0.17	0
Theophylline (μg/ml)						
0	1.00	100	1.00	100	1.00	100
156	1.01	86	1.05	139	1.00	100
313	1.03	93	0.89	117	0.96	99
625	0.88	55	1.00	128	0.83	101
1250	0.75	44	1.08	158	0.82	63
2500	0.88	77	1.01	109	0.52	40
5000	0.87	74	1.11	111	0.45	34
Amino Acid Copper Complex (%)						
0	1.00	100	1.00	100	1.00	100
0.313	0.15	0	0.20	17	0.13	0
0.625	0.14	0	0.05	3	0.20	0
1.25	0.15	0	0.03	0	0.18	0
2.5	0.09	0	0.04	0	0.17	0
5	0.10	0	0.02	0	0.21	0
10	0.04	0	0.08	0	0.15	0

Following adjustment of the cultures to 3×10^5 cells/ml after treatment, cells were incubated at 37°C in a humidified 5% CO_2/95% air incubator for 2 days. On day 1, cell numbers were adjusted, after counting, to 3×10^5 cells/ml. The small sample taken at the end of the treatment period were diluted to 8 cells/ml. 0.2 ml of the final concentration of each culture is placed into each well of 96-well microtiter plates. The plates were incubated at 37°C for 9 days. Wells containing viable clones were identified and counted by eye.
[a]Relative Suspension Growth.
[b]% Relative Total Growth.

S9 mix and 31.3 to 250 μg/ml with S9 mix. Amino acid copper complex was more toxic, causing 100% cytotoxicity even at lowest concentration of 0.313% without S9 mix. Therefore, 0.0625% was selected as the highest concentration without S9 mix. Preliminary assay also showed that the highest concentration that could be achieved with amino acid copper complex with S9 mix was 0.25% because the chemical showed 17% RTG at concentration of 0.313%. Conversely, theophylline exhibited no cytotoxic effects at any concentrations at all. For relatively non-cytotoxic test chemicals, 5 mg/ml was determined as the highest concentration. Thus, 5 mg/ml was selected as the highest concentration for MLA of theophylline. For mutation experiment, test chemicals of highest concentration was serially twofold diluted to make 4 dose levels, and then assayed in duplicate per dose.

2. Mouse lymphoma cell mutation assay with hydroxyurea

Hydroxyurea, which is known to inhibit DNA replication (Anand *et al.*, 1995), was classed positive in the absence of S9 mix (Table 2). When cells were exposed to hydroxyurea for 4 h without S9 mix, mutant frequency was gradually increased at 125 to 500 μg/ml of hydroxyurea. There was also statistically significant increase in mutant frequency (MF) in the absence of a linear trend when the exposure period without S9 mix was increased to 24 h. Analysis of colony sizing showed that hydroxyurea induced predominantly small colonies, indicating large-scale chromosomal deletions. Significant increases in MF were induced by NQO, the positive control without S9 mix, which increased the frequency of both the small and large colonies. In contrast, in the experiment with S9

Table 2. Cloning data for L5178Y/TK+/- mouse lymphoma cells with hydroxyurea

Conc. (μg/ml)	Survival (%RS)[a]	Viability (%RTG)[b]	RSG[c]	Mutant frequency				
				$PE_{(viable)}$[d]		$PE_{(mutant)} \times 10^{-5}$		MF
				A	B	A	B	
4 hr (-S9)								
0	100	100	1.00	0.32	0.60	4.92	7.28	139
125	86	108	1.12	0.49	0.38	6.68	11.70	221
250	61	88	0.95	0.37	0.47	11.10	7.28	225
500	54	64	0.99	0.15	0.29	7.28	12.30	458
1000	60	50	0.95	0.24	0.23	8.49	4.35	267
NQO 0.1	76	46	0.79	0.26	-	14.40	-	548
4 hr (+ S9)								
0	100	100	1.00	0.19	0.15	2.67	2.13	144
62.5	78	93	0.87	0.23	0.18	1.59	3.23	125
125	100	100	0.93	0.30	0.18	4,92	2.13	140
250	123	108	1.11	0.13	0.20	1.59	3.23	143
500	132	87	1.12	0.15	0.11	2.13	1.59	143
BP 3	57	35	0.51	0.11	-	1.59	-	139
24 hr (- S9)								
0	100	100	1.00	0.73	0.71	3.79	6.08	69
31.3	112	91	1.03	0.50	0.77	6.08	10.40	128
62.5	84	60	0.80	0.53	0.55	12.30	10.40	211
125	60	65	0.78	0.60	0.60	4.35	7.88	132
250	19	18	0.38	0.24	0.43	7.28	12.30	292
NQO 0.04	39	7	0.38	0.13	-	15.10	-	1161

Following adjustment of the cultures to 3×10^5 cells/ml after treatment, cells were incubated at 37°C in a humidified 5% CO_2/ 95% air incubator for 2 days. On day 1, cell numbers were adjusted, after counting, to 3×10^5 cells/ml. On day 2 cell counts were determined. For plating efficiency assay, each culture was diluted to 8 cells/ml and cultured in 96 well microtiter plates for 9 days. For mutant selection assay, each culture was diluted to 1×10^4 cells/ml and cultured in the presence of TFT in 96 well microtiter plate for 12 days. For survival assay, the small sample taken at the end of the treatment period were diluted to 8 cells/ml. 0.2 ml of the final concentration of each culture is placed into each well of 96-well microtiter plates. The plates were incubated at 37°C for 9 days. Wells containing viable clones were identified and counted by eye.
A and B are duplicate cultures. BP : Benzo(a)pyrene, NQO : 4-Nitroquinoline-1-oxide.
[a]Relative Survival.
[b]% Relative Total Growth.
[c]Relative Suspension Growth.
[d]Plating Efficiency.

mix, hydroxyurea-induced MF increase was not observed. Unfortunately, there was not MF increase in cultures with benzo(a)pyrene used as a positive control in the presence of S9 mix. Therefore, the results with S9 mix did not meet the acceptance criteria for MLA.

3. Mouse lymphoma cell mutation assay with theophylline

Theophylline, which is commonly known as xanthine derivatives, is generally used as central nervous system stimulators (Persson, 1986). As shown in Table 3, treatment of cells with theophylline for 4 h or 24 h did not lead to increased MF without S9 mix. Conversely, with S9 mix, theophylline resulted in an increase in MF (3.3 times that of the average negative control value) at 1250 μg/ml after 4 h treatment. However, theophylline was concluded to be negative because only cultures treated with 1250 μg/ml theophylline could be evaluated for mutagenicity and a dose-response trend was not observed. In the positive control, there was a clear increase in mutant frequency (2.3~3.5 times that of the average negative control value at different conditions), indicating that the S9 mix was active and the test system was capable of detecting a mutagen.

4. Mouse lymphoma cell mutation assay with amino acid copper complex

Amino acid copper complex, which was positive in chromosomal aberration test (CA) of our previous study, did not induce mutants at all regardless of S9 mix at 4 h treatment experiment (Table 4). In contrast, when treated at the concentrations of 0.0156%

Table 3. Cloning data for L5178Y/TK+/- mouse lymphoma cells with theophylline

Conc. (μg/ml)	Survival (%RS)[a]	Viability (%RTG)[b]	RSG[c]	Mutant frequency				
				$PE_{(viable)}$[d]		$PE_{(mutant)} \times 10^{-5}$		MF
				A	B	A	B	
4 hr (-S9)								
0	100	100	1.00	0.23	0.25	2.13	1.59	77
625	85	81	0.92	0.20	0.23	2.67	-	136
1250	112	79	0.75	0.35	0.16	2.67	1.59	88
2500	95	92	0.88	0.24	0.26	3.23	1.05	86
5000	83	79	0.87	0.19	0.25	3.23	1.59	117
NQO 0.1	26	35	0.86	0.10	-	2.67	-	270
4 hr (+ S9)								
0	100	100	1.00	0.33	0.24	3.23	3.23	116
625	75	82	0.99	0.20	0.27	-	5.50	201
1250	100	78	1.18	0.18	0.20	8.49	6.08	390
2500	90	95	1.28	0.20	0.26	4.35	3.23	172
5000	121	227	1.77	0.37	0.36	4.92	6.08	151
BP 3	39	35	0.99	0.10	-	2.67	-	270
24 hr (- S9)								
0	100	100	1.00	0.40	0.30	5.50	4.92	151
625	89	92	0.93	0.36	0.34	-	3.79	112
1250	100	111	1.21	0.36	0.28	3.23	-	90
2500	60	43	0.72	0.30	0.12	4.35	2.67	184
5000	37	24	0.64	0.12	0.14	-	2.67	193
NQO 0.04	26	8	0.60	0.05	-	1.59	-	338

Following adjustment of the cultures to 3×10^5 cells/ml after treatment, cells were incubated at 37°C in a humidified 5% CO_2/95% air incubator for 2 days. On day 1, cell numbers were adjusted, after counting, to 3×10^5 cells/ml. On day 2 cell counts were determined. For plating efficiency assay, each culture was diluted to 8 cells/ml and cultured in 96 well microtiter plates for 9 days. For mutant selection assay, each culture was diluted to 1×10^4 cells/ml and cultured in the presence of TFT in 96 well microtiter plate for 12 days. For survival assay, the small sample taken at the end of the treatment period were diluted to 8 cells/ml. 0.2 ml of the final concentration of each culture is placed into each well of 96-well microtiter plates. The plates were incubated at 37°C for 9 days. Wells containing viable clones were identified and counted by eye.
A and B are duplicate cultures. BP : Benzo(a)pyrene. NQO : 4-Nitroquinoline-1-oxide.
[a]Relative Survival.
[b]% Relative Total Growth.
[c]Relative Suspension Growth.
[d]Plating Efficiency.

Table 4. Cloning data for L5178Y/TK+/- mouse lymphoma cells with amino acid copper complex

Conc. (%)	Survival (%RS)[a]	Viability (%RTG)[b]	RSG[c]	Mutant frequency				
				$PE_{(viable)}$[d]		$PE_{(mutant)} \times 10^{-5}$		MF
				A	B	A	B	
4 hr (-S9)								
0	100	100	1.00	0.41	0.46	7.27	6.68	162
0.0078	79	103	0.95	0.46	0.47	7.28	11.00	195
0.0156	53	103	0.89	0.49	0.52	5.50	2.13	113
0.0313	52	88	0.88	0.47	0.40	4.35	5.50	137
0.0625	39	70	0.82	0.42	0.32	7.88	7.28	209
NQO 0.1	52	69	0.86	0.35	-	21.90	-	629
4 hr (+ S9)								
0	100	100	1.00	0.46	-	6.68	-	145
0.0313	100	82	0.92	0.41	-	4.35	-	107
0.0625	77	76	0.97	0.30	0.42	3.79	4.35	115
0.125	28	29	0.70	0.24	0.15	2.67	2.67	146
0.25	33	42	0.83	0.25	0.22	4.35	3.23	161
BP 3	38	55	0.92	0.27	-	7.88	-	289
24 hr (- S9)								
0	100	100	1.00	0.67	0.79	7.88	9.11	117
0.0078	9	4	0.20	0.15	0.73	1.05	9.74	101
0.0156	5	2	0.14	0.11	0.05	3.37	1.05	260
0.0313	6	1	0.09	0.11	0.05	2.67	1.05	223
0.0625	7	3	0.11	0.14	-	2.13	-	154
NQO 0.04	43	24	0.39	0.45	-	15.10	-	339

Following adjustment of the cultures to 3×10^5 cells/ml after treatment, cells were incubated at 37°C in a humidified 5% CO_2/95% air incubator for 2 days. On day 1, cell numbers were adjusted, after counting, to 3×10^5 cells/ml. On day 2 cell counts were determined. For plating efficiency assay, each culture was diluted to 8 cells/ml and cultured in 96 well microtiter plates for 9 days. For mutant selection assay, each culture was diluted to 1×10^4 cells/ml and cultured in the presence of TFT in 96 well microtiter plate for 12 days. For survival assay, the small sample taken at the end of the treatment period were diluted to 8 cells/ml. 0.2 ml of the final concentration of each culture is placed into each well of 96-well microtiter plates. The plates were incubated at 37°C for 9 days. Wells containing viable clones were identified and counted by eye.
A and B are duplicate cultures. BP : Benzo(a)pyrene, NQO : 4-Nitroquinoline-1-oxide.
[a]Relative Survival.
[b]% Relative Total Growth.
[c]Relative Suspension Growth.
[d]Plating Efficiency.

Table 5. Comparison of the known CA results and the MLA results in the present study

Drug	MLA	*in vitro* CA
Hydroxyurea	Positive	Positive (Sherwood *et al.*, 1988)
Theophylline	Negative	Positive (Ishidate *et al.*, 1987)
Amino acid copper complex	Equivocal	Positive (KIT-KRICT report)

and 0.0313% for 24 h, the mutant fraction were 2.2 and 1.9 times over negative control value ($117/10^6$ cells). However, 0.0156% and 0.0313% were excluded from the statistical analysis as %RS and %RTG was below the acceptable minimum of 10%. Due to the higher toxicity, %RTG was 4% even at the lowest concentration, 0.0078%. Therefore, amino acid copper complex was concluded to be equivocal under the condition of this study. There was a clear increase in mutant frequency in the positive control (2.0~3.9 times that of the average negative control value at different conditions).

In conclusion, among 3 CA-positive chemicals, positive MLA result was obtained for hydroxyurea. The other two chemicals, theophylline and amino acid copper complex, were negative and equivocal (Table 5).

IV. DISCUSSION

In this study, mouse lymphoma assay (MLA) was performed with three test chemicals (hydroxyurea, theophylline and amino acid copper complex), which were reportedly positive in the chromosomal aberration test. When the cells were exposed to test chemi-

cals for 4 h without S9 mix, positive MLA result was obtained for hydroxyurea while theophylline and amino acid copper complex were negative. Although the 24 h treatment is not specifically recommended in the OECD Guidelines (OECD, 1997), Honma *et al.* (1999b) reported that short treatments (3~6 h) may be insufficient for detecting some clastogens and spindle poisons and the sensitivity of the MLA with 24 h treatment approaches that of the chromosome aberration test. However, treatment of cells with theophylline for 24 h did not lead to increased MF either. In contrast, amino acid copper complex was concluded to be equivocal at 24 h treatment experiment. Although the mutant fraction were 2.2 and 1.9 times over negative control value at concentrations of 0.0156% and 0.0313%, those concentrations were excluded from the statistical analysis as %RS and %RTG was below the acceptable minimum of 10%. The biologically irrelevant effects might occur in severely stressed cells (Scott *et al.*, 1991). Therefore, the lower top concentration showing 10~20% RTG should have been used at 24 h treatment with amino acid copper complex. However, it is extremely difficult to achieve such a narrow window. Kirkland *et al.* (1998) reported that concentration spacing often needs to be closer than obtained with a 2-fold dilution series, and a dilution factor where each concentration is 0.75 or 0.8 of the one above is often required when testing toxic chemicals. On the other hand, RTG was used for preliminary dose-finding in this study. Generally, two parameters, RS and RTG, are currently widely used for evaluating cytotoxicity for concentration range experiments and selection of concentrations for full mutation assay. RS is determined by cloning the cells immediately after treatment while RTG takes into account cell loss after treatment, reduction in growth rate over the expression period, and any reduction in cloning efficiency on the day of selection for mutants (Moore *et al.*, 2000). Traditionally, the relative survival (RS) by day 0 plating efficiency was used for the microwell method and the relative total growth (RTG) was used for the agar plate method. However, for some chemicals such as vinblastine, no decrease of the RS was seen at any dose level, but the RTG decreased remarkably as the dose increased (Sofuni *et al.*, 1997). Especially, for chemicals that block cell cycle progression, such as hydroxyurea, RTG may give a better assessment of cytotoxicity than the RS.

The recommended acceptable range of spontaneous mutant frequency is below $60/10^6$ cells. Our spontaneous mutations, which was 69~162, was higher than recommended level. However, there was no statement about an acceptable upper limit. Moreover, a large interlaboratory variation is occasionally seen: the lowest value was $23/10^6$ cells and the highest one was $628/10^6$ cells. At present, 90% of the individual data showed the range of $50~250/10^6$ cells in spontaneous mutations (Sofuni *et al.*, 1997). Therefore, it is considered that spontaneous mutation value obtained in this study is acceptable range for the negative control.

In addition to detecting chromosome damage, such as chromosome aberration test, MLA is able to discriminate the chemical's ability to induce gene mutations and chromosome mutations. Mutant colonies detected in MLA can be classified by colony size as large and small resulting from different degrees of genetic damage. Although the molecular difference between large and small colony mutants remains unknown. it is assumed to be due to a putative growth control gene distal but very close to tk gene (Applegate *et al.*, 1990). Especially, small colony mutants with slow growth kinetics may result from loss of a putative growth control gene, differential mechanisms of chromosome breakage/repair and second site mutations (Moore *et al.*, 1985; Hozier *et al.*, 1981; Blazak *et al.*, 1989; Liechty *et al.*, 1998). The small colonies are defined as less than a quarter of the diameter of the well and have compact morphology (Honma *et al.*, 1999a). Hydroxyurea, which was classed positive without S9 mix, induced predominantly small colonies. Therefore, colony sizing analysis suggested that hydroxyurea caused large-scale chromosomal deletions.

In conclusion, among three CA-positive test chemicals, positive MLA results was obtained for one, indicating that CA yielded higher sensitivity than MLA. However, MLA, which evaluates mutagenic potential of chemicals through colony formation by cell growth, may provide a higher predictivity of carcinogenesis than CA. In fact, *P*-values for the Fischer's exact test for each assay result as predictors of carcinogenicity were: CA, 0.72; MLA 0.25, indicating the relative strength of the MLA compared to CA (Snyder *et al.*, 2001). Moreover, MLA has higher specificity (the ability of a test to predict non-carcinogenicity) and positive pre-

dictivity (the proportion of chemicals accurately predicted to be carcinogens by a particular test) than CA. Especially, Mitchell *et al.* (1997), in an extensive review of the published MLA and rodent carcinogenicity literature, have concluded that, for those chemicals that yielded clear-cut results in both assays, the MLA and the rodent carcinogenicity bioassay are in excellent agreement with each other. In addition, assessment of the relative numbers of both colony types can provide information to support results obtained in bacterial reverse mutation and CA.

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