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Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the *in vitro* chromosomal aberration test

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In order to evaluate the utility of the mouse lymphoma assay (MLA) for detecting *in vitro* clastogens and spindle poisons and to compare it with the *in vitro* chromosomal aberration test (CA), we conducted an international collaborative study of the MLA that included 45 Japanese laboratories and seven overseas laboratories under the cooperation of the Ministry of Health and Welfare of Japan and the Japanese Pharmaceutical Manufacturer's Association. We examined 40 chemicals; 33 were reportedly positive in the CA but negative in the bacterial reverse mutation assay, six were negative in both assays and one was positive in both. We assayed mutations of the thymidine kinase (TK) locus (*tk*) of L5178Y $tk^{+/-}$ mouse lymphoma cells using the microwell method. According to our standard protocol, cells were exposed to the chemical for 3 h, cultured for 2 days and TK-deficient mutants were expressed in 96-well plates under trifluorothymidine. Each chemical was coded and tested by two or three laboratories. Among the 34 CA-positive chemicals, positive MLA results were obtained for 20 and negative results were obtained for nine. The remaining five chemicals were inconclusive or equivocal because of discrepant inter-laboratory results or reproduced discrepant results, respectively. Among the six CA-negative chemicals, one was negative in the MLA, two were positive and three were inconclusive. Thus, the MLA could detect only 59% (20/34) of CA-positive chemicals. We concluded that the MLA was not as sensitive as the CA. Some MLA-negative chemicals evoked positive responses in the CA only after long continuous treatment. These might also be genotoxic in the MLA with long continuous treatment. Improvement of the MLA protocol, including alteration of the duration of the treatment, might render the MLA as sensitive as the CA.

Introduction

The genotoxicity tests that have been developed and validated over the years differ in the biological system used (prokaryotic, eukaryotic, *in vitro* and *in vivo*) and the endpoints detected (gene mutation, chromosome aberration, DNA damage, etc.). Because no single test is capable of detecting all genotoxic chemicals, a battery of tests covering different targets and endpoints and using different systems should be used to ensure that the majority of genotoxic chemicals can be identified. Although this philosophy has been agreed upon internationally, the make-up of the battery differs among countries and organizations. In the Japanese guidelines for the testing of pharmaceuticals established in 1989 (Ishidate, 1988; MHW Japan, 1990), the minimum test battery consists of a bacterial reverse mutation assay (BRM), an *in vitro* chromosome aberration assay (CA) and an *in vivo* rodent micronucleus assay (MN). The European guidelines recommend four tests, i.e. a BRM, a CA, an *in vitro* mammalian cell gene mutation assay (MCGM) and an *in vivo* test (CEC/EU, 1989; DHSS UK, 1989). The US Food and Drug Administration (FDA) has no written guidelines for pharmaceuticals, but the agency's Centers for Drugs and Biologics Evaluation and Research applies to pharmaceuticals the guidelines on genetic toxicity testing provided by the FDA Center for Food Safety and Applied Nutrition (Federal Register, 1993); it requires a three or four test battery with preferential inclusion of a mouse lymphoma assay (MLA). The three test battery consists of a BRM, a MLA and an *in vivo* cytogenetic test (metaphase analysis or MN) and the four test battery includes a MCGM and a CA instead of the MLA. The US Environmental Protection Agency (EPA) recommends that mutagenicity testing of pesticides and toxic substances also begin with the three test battery including the MLA (Dearfield, 1989; Dearfield *et al*., 1991).

In an effort to harmonize genotoxicity testing internationally, standardization of the genotoxicity test battery is being discussed by the Expert Working Group on Genotoxicity of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) (ICH, 1994, 1996). A pivotal issue is whether the MCGM assay, in particular the MLA, is necessary in the standard battery. The MLA quantifies genetic alterations affecting expression of the thymidine kinase (TK) gene (*tk*). This assay was developed by Drs D.Clive and M.Moore (Clive and Spector, 1975; Clive *et al*., 1979; Moore-Brown *et al*., 1981) and the protocol has been optimized (Moore and Clive, 1982; Moore and Howard, 1982; Cole *et al*., 1983; Turner *et al*., 1984; Majeska and Matheson, 1990). Some TK-deficient mutants in the MLA exhibit not only point mutations but also gross structural and numerical changes at the chromosomal level (Hozier *et al*., 1982; Moore *et al*., 1985; Blazak *et al*., 1989; Clive *et al*., 1990; Combes *et al*., 1995; Zhang *et al*., 1996), thus the MLA can detect a wide range of genetic damage, including gene mutations, larger scale chromosomal changes, recombination, aneuploidy and others. Most of these changes occur in tumors and are presumably relevant for carcinogenesis. That is why US regulatory authorities advocate

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inclusion of the MLA rather than the CA into the standard genotoxic test battery (Tennant *et al*., 1987; Garriot *et al*., 1995). On the other hand, although the MLA is sensitive, its specificity is not high (Tennant *et al*., 1987; Mitchel *et al*., 1997) and the test is unreliable when marginal or weak positive responses are associated with excessive toxicity (Li *et al*., 1991; Caldwell, 1993).

One critical issue is whether the MLA can detect *in vitro* clastogens and spindle poisons as well as the CA can. To determine whether the MLA is a suitable alternative to the CA, we conducted a two-phase international collaborative study under the auspices of the Ministry of Health and Welfare of Japan and the Japanese Pharmaceutical Manufacturer's Association. We published an interim report after the phase 1 study (Sofuni *et al*., 1996). This, the final report, includes the first and second phase results, an overall evaluation of the MLA sensitivity relative to the CA and some protocol issues.

Study design

The major aim of this collaborative study was to evaluate the MLA for inclusion in a genotoxicity test battery. To assess the assay's sensitivity relative to the CA, we chose chemicals that were mostly positive in the CA but negative in the BRM. The first phase of the study was conducted by 42 Japanese and seven overseas laboratories during September 1994–March 1995. The second phase was conducted by 39 Japanese and six overseas laboratories during May 1995–November 1995. Participating laboratories are listed in Table I. The chemicals tested in the first and second phases are listed in Table II and III, respectively. The published results of other *in vitro* genotoxicity tests of the same chemicals are also shown.

In the phase 1 study, 20 chemicals were tested and all but one (urethane) were positive in the CA and negative in the BRM. Six of the chemicals (bromodichloromethane, chlorendic acid, chlorodibromomethane, isophorone, pentachloroethane and tetrachloroethane) had been reported as CA-negative by the National Toxicology Program (NTP) (Zeiger *et al*., 1990), but re-evaluation using CHL/IU cells and Japanese guidelines indicated that the chemicals were positive (Matsuoka *et al*., 1996) and we classified them as such. Mitomycin C, being positive in both the BRM and CA, was used as an internal positive control.

In the phase 2 study, we used 23 chemicals; 14 were positive in the CA but negative in the BRM, five were negative in both assays, one was positive in both and three chemicals (bromodichloromethane, isophorone and tetrachloroethane) had yielded inconclusive results in the phase 1 study.

In total, therefore, we studied 40 chemicals, 34 of which were reportedly positive in the CA. Of these, eight (*N*-aminoethyl ethanolamine, *p*-t-butylphenol, chlorodibromomethane, diethylstilbestrol, noscapine, tetrachloroethane, thiabendazole and pentachloroethane) were reported to induce mainly numerical chromosome changes. Each test chemical was tested by two or three laboratories with and without S9 mix to confirm the results and to evaluate inter-laboratory variability. We employed the microwell method because it is well validated, free from agar quality problems and has easier detection of small colonies than the agar method (Cole *et al*., 1983, 1991; Clay and Cross, 1990).

Materials and methods

Cell culture and maintenance

L5178Y $tk^{+/-}$ clone 3.7.2C mouse lymphoma cells were obtained from Dr D.Clive (Glaxo Welcome Co., Research Triangle Park, NC) and expansively cultured to make master stocks. Master stocks were maintained in liquid nitrogen at a density of 1×10^6 cells/ml, 1 ml/tube, in culture medium containing 5% dimethylsulfoxide (DMSO). They were confirmed as free from mycoplasma by a Hoechst staining technique. The master stocks were distributed to each laboratory participating in the collaborative study. Each laboratory thawed the master stock and expansively cultured the cells for use and for laboratory stocks. Cell density was determined with a hemocytometer or an automatic cell counter and the cultures were routinely diluted to ~2×10^5 cells/ml each day to prevent overgrowth (>10^6 cells/ml). Logarithmic growth was normally maintained with population doubling times of 9–11 h. To prepare working stocks for gene mutation experiments, cultures were purged of $tk^{-/-}$ mutants by exposure for 1 day to THMG medium (culture medium containing 3 μg/ml thymidine, 5 μg/ml hypoxanthine, 0.1 μg/ml methotrexate and 7.5 μg/ml glycine) and then the cells were transferred to THG medium (THMG but without methotrexate) for 2 days. The purged cultures were checked for low background $tk^{-/-}$ mutants and stored in liquid nitrogen. Each experiment started with working stock. The cells were usually used on day 3 or 4 after thawing and during logarithmic growth.

Media

RPMI 1640 medium (catalogue no. 31800; Gibco BRL Life Technologies Inc., Grand Island, NY) and three lots of donor horse serum (lot nos. 4001213 and 4002048; BioCell Laboratories Inc., Rancho Dominguez, CA; lot no. MB02F007; Intergen Company, Purchase, NY) were generally used in all laboratories except for a few overseas laboratories. The horse serum was inactivated at 56°C for 30 min.

Basic medium (designated $RPMI_0$) consisted of RPMI 1640 medium supplemented with 200 μg/ml sodium pyruvate, 0.5 mg/ml pluronic, 100 U/ml penicillin and 100 μg/ml streptomycin. Growth medium (designated $RPMI_{10}$) was $RPMI_0$ with 10% (v/v) heat-inactivated horse serum. The cells were treated with the test chemicals in basic medium with 5% serum. Cloning medium for colony formation in 96-well plates was basic medium with 20% serum and without pluronic ($RPMI_{20}$).

S9 activation condition

A single lot of post-mitochondrial supernatant fractions of rat liver homogenates (S9) was purchased from Kikkoman Co. Ltd (Noda, Chiba, Japan) and distributed to each laboratory. The S9 had been made from the liver of phenobarbital- and 5,6-benzoflavone-pretreated Sprague–Dawley rats. S9 mix was prepared just prior to use by combining 4 ml S9 with 2 ml each 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP and 150 mM KCl. The concentration of S9 mix was 5% during treatment and the final concentration of S9 was 2%.

Test chemicals

2′-Deoxycoformycin and cinnamyl anthranilate were gifts from Drs T.Shigaki (The Chemosero Therapeutic Research Institute, Japan) and M.Shelby (NIEHS, Research Triangle Park, NC), respectively. The other test chemicals were supplied by Wako Pure Chemical Co. Ltd (Osaka, Japan). Chemicals were coded for the study and stored at 4 or –20°C. Just prior to each experiment, an aliquot was placed in an appropriate solvent and diluted as appropriate for the treatment of cells in suspension (the maximum was 10% for aqueous solutions and 1% for organic solutions).

Solvent (vehicle)

The solvents used, in order of preference, were medium without serum ($RPMI_0$), physiological saline, distilled water or DMSO. If the test chemical could not be dissolved in any solvent, it was suspended in $RPMI_0$.

Negative and positive control

Negative and positive control cultures were included in each experiment. The test chemical solvent was used for the negative controls. The positive control chemical was 10 μg/ml methylmethansulfonate (MMS; Aldrich Chemical Co. Inc., Milwaukee, WI) in experiments without S9 mix and 3 μg/ml cyclophosphamide (CP; Sigma Chemical Co., St Louis, MO) in experiments with S9 mix. MMS and CP were stored at 4°C and a fresh solution was prepared with physiological saline for each experiment (1 and 0.3 mg/ml, respectively).

Experimental design

We conducted preliminary experiments to determine the solubility and cytotoxicity of the test chemicals. Cytotoxicity was determined by relative survival (RS) and relative total growth (RTG) following 3 h treatments at concentrations up to 5 mg/ml, usually regardless of solubility. The recommended highest concentration was one with a 10–20% RS and/or RTG. There was no perceived need to test concentrations >5 mg/ml.

Each main experiment usually consisted of one solvent control, one positive control and at least three concentrations of the test chemical. As a rule, 2-fold serial dilutions were prepared from the highest concentration. All

Table I. Participants in the MLA collaborative study

No.	Laboratory	Investigators
1	An-Pyo Center	T.Kitazawa, M.Itakura, M.Nakajima
2	BML Inc.	F.Yokota
3	Centre International de Toxicologie (France)	B.Molinier, S.de Jouffrey
4	Chemical Inspection & Testing Institute	T.Kikuno
5	Covance Laboratories Ltd (UK)	J.Clements
6	Daicel Chemical Industries Ltd	T.Baba, S.Kitamura, A.Yamazaki
7	Daiichi Pharmaceutical Co. Ltd	S.Nakayama, H.Shimada
8	Food and Drug Safety Center	S.Wakuri, N.Tanaka
9	Fuji Biomedix Co. Ltd	T.Inoue
10	Fujisawa Pharmaceutical Co. Ltd	A.Miyamoto
11	Glaxo Wellcome Research and Development (UK)	P.Wilcox, J.Oliver
12	Institute of Applied Medicine (HRIC) Ltd	S.Hashimoto, M.Tada
13	Institute of Environmetal Toxicology	H.Matsumura, K.Matsumoto
14	Institute Pasteur de Lille (France)	D.Marzin
15	Japan Bioassay Research Center	M.Asakura, T.Sugiyama
16	Japan Tobacco Inc.	M.Taketomi
17	JBC Co. Ltd	O.Suzuki
18	Kaken Pharmaceutical Co. Ltd	J.Yoshida
19	Kyorin Pharmaceutical Co. Ltd	Y.Kasahara
20	Kyowa Hakko Kogyo Co. Ltd	K.Tanaka
21	Meiji Seika Kaisha Ltd	H.Hayashi
22	Microbiological Associates Inc.(USA)	R.H.C.San, D.L.Putman
23	Mitsubishi Chemical Co.	Y.Iwase
24	Mitsubishi Chemical Safety Institute Ltd	M.Nakagawa, T.Nishitomi
25	Mitsui Pharmaceutical Co. Ltd	H.Misono
26	Division of Environmental Chemistry, National Institute of Health Sciences	T.Nishimura
27	Division of Genetics and Mutagenesis, National Institute of Health Sciences	M.Honma, M.Hayashi, T.Sofuni
28	Nihon Bioresearch	Y.Miwa
29	Nippon Glaxo Ltd	T.Morita
30	Nippon Menard Cosmetic Co. Ltd	H.Kojima
31	Nippon Shinyaku Co. Ltd	Y.Yamashita, H.Tamura, N.Sumi
32	Nitto Denko Corp.	N.Asano
33	Olympus Optical Co. Ltd	K.F.Miura, M.Ishidate Jr.
34	Otsuka Pharmaceutical Co. Ltd	T.Awogi, Y.Ohara, M.Tanaka
35	Otsuka Pharmaceutical Factory Inc.	K.Shimono
36	Rhone-Poulenc Rorer (France)	M.C.Ouldelhkim, V.Thyband
37	Sankyo Co. Ltd	K.Hirano, T.Hasegawa
38	Shionogi & Co. Ltd	Y.Miyake, H.Miyajima
39	Shiseido Co. Ltd	H.Kobayashi, M.Mori
40	Snow Brand Milk Products Co. Ltd	N.Yajima, M.Kawabata
41	SRI International (USA)	C.Rudd
42	SS Pharmaceutical Co. Ltd	S.Hamada
43	Sumitomo Chemical Co. Ltd	S.Kitamoto
44	Taiho Pharmaceutical Co. Ltd	H.Oka, A.Ohuchida
45	Takeda Chemical Industry Ltd	N-U.Kodani, K.I.Yamamoto
46	Tanabe Seiyaku Co. Ltd	S.Nitou, Y.Kondo
47	Teijin Ltd	Y.Kasahara, Y.Takahashi
48	Tokyo Tanabe Co. Ltd	H.Daigo
49	Toyama Chemical Co. Ltd	S.Nakamura
50	Yamanouchi Pharmaceutical Co. Ltd	K.Yuno, A.Wakata
51	Yoshitomi Pharmaceutical Industry Ltd	H.Hirono, M.Takeuchi, E.Yamamura
52	Zeria Pharmaceutical Co. Ltd	A.Matsui, K.Shibata

chemicals were tested with and without S9 mix. In the phase 1 study all experiments were carried out in duplicate according to the UKEMS guidelines (Cole *et al*., 1990), while most of the experiments in the phase 2 study used single cultures.

Mutation experiments

Chemical treatment. Ten million cells in 10 ml $RPMI_{10}$ medium were placed in each of a series of sterile disposable 50 ml centrifuge tubes. Solvent, test chemical or positive control solution and 1 ml S9 mix or 150 mM KCl solution were added. The total volume of each tube was adjusted to 20 ml by addition of $RPMI_0$ medium.

The cells were incubated with gentle shaking on a rocker platform in an incubator at 37°C for 3 h. After treatment, the cells were collected by centrifugation and washed once with $RPMI_0$. The cells were then resuspended in 50 ml $RPMI_{10}$ at ~2×10^5 cells/ml. Cells were counted with a hemocytometer or an automatic cell counter. The cells were transferred to flasks for growth through the expression period or diluted to be plated for survival as described below.

Plating for survival. An aliquot of each culture was diluted to 8 cells/ml with $RPMI_{20}$. It was placed into 96-well plates at 0.2 ml/well (two plates for solvent control and one plate each for test chemical treatment and positive control). The plates were incubated at 37°C in a humidified incubator gassed with 5% CO_2 in air. Colonies formed in the individual wells were assessed after 11–13 days.

Expression period. Cultures were maintained in flasks without shaking for 45 h, which was required for expression of the TK-deficient phenotype. During this period, cell densities were controlled at 10^5–10^6 cells/ml. Cell densities were monitored on day 1 and then cells were diluted to ~2×10^5 cells/ml. On completion of the 45 h expression time (day 2), cell densities were counted again and relative suspension growth (RSG) was calculated from the cell counts on days 1 and 2.

Plating for viability. The cultures were adjusted to 104 cells/ml with $RPMI_{20}$ at volumes of 100 ml for solvent control and 50 ml for test chemical treatments and positive control in readiness for plating for 5-trifluorothymidine (TFT) resistance. A portion of each culture was removed and diluted to 8 cells/ml.

Table II. Chemicals tested in the phase 1 study and previously published *in vitro* genotoxicity data

Chemical name	CAS no.	Mol. wt	CA			BRM		MCGM	
			Structural −S9/+S9	Numerical −S9/+S9	Reference		Reference		References
Arsenic trioxide	1327-53-3	197.8	+/		Nakamuro and Sayoto (1981)	−	Jacobson-Kram and Montalbano (1985)		
Benzene	71-43-2	78.1	−/+	−/−	Ishidate (1987)	−	Shimizu *et al.* (1983)	−	Oberly *et al.* (1984)
Bromodichloromethane	75-27-4	163.8	−/+	−/−	Matsuoka *et al.* (1996)	−	Mortelmans *et al.* (1986)	+	McGregor *et al.* (1988a)
Cadmium sulphate	10124-36-4	208.5	+/	+/	Bean *et al.* (1992)	−	Marzin and Phi (1985)	+	Oberly *et al.* (1982)
Chlorendic acid	115-28-6	388.3	−/+	−/−	Matsuoka *et al.* (1996)	−	Haworth *et al.* (1983)	+	McGregor *et al.* (1988a)
Chlorodibromomethane	124-48-1	208.3	−/−	+/−	Matsuoka *et al.* (1996)	−	Zeiger *et al.* (1987)	+	McGregor *et al.* (1991)
Cytosine arabinoside (Ara C)	147-94-4	243.3	+/	−/	Ishidate (1987)	−	Zeiger *et al.* (1987)	+	McGregor *et al.* (1989)
Dideoxycytidine (DDC)	7481-89-2	811.1	+/+	−/−	Chetelat (1987)	−	PDR (1995)	−	PDR (1995)
Diethylstilbestrol (DES)	56-53-1	268.4	−/	+L/	Ishidate (1987)	−	Anderson and Styles (1978)	−, +	Amacher and Zelljadt (1984), Myhr and Caspary (1988)
Eugenol	97-53-0	164.0	+L/	−/	Ishidate (1987)	−	Sekizawa and Shibamoto (1982)		
5-Fluorouracil (5-FU)	51-21-8	211.3	+/	−/	Ishidate (1987)	−	Seino *et al.* (1978), Yajima *et al.* (1981)		
Griseofulvin	126-07-8	352.8	+L/	+L/	Larizza *et al.* (1974)	−	Wehner *et al.* (1978b)		
Hexamethyl phosphoramide	680-31-9	179.2	+/	−/	Ishidate (1987)	−	Ashby *et al.* (1985)	−, +	Suter *et al.* (1992), Jotz and Mitchell (1981)
Hydroxyurea	127-07-1	76.1	+/		Sherwood *et al.* (1988)	−	Bruce and Heddle (1979)	+	Garberg *et al.* (1988)
Isophorone	78-59-1	138.2	+/+	−/−	Matsuoka *et al.* (1996)	−	Mortelmans *et al.* (1986)	−, +	O'Donoghue *et al.* (1988), McGregor *et al.* (1988b)
Methotrexate (MTX)	59-05-2	454.5	+/		Mondello *et al.* (1984)	−	Seino *et al.* (1978)	−, +	Dickins *et al.* (1985), Clive *et al.* (1979)
Monocrotaline	315-22-0	325.4	−/+		Umeda and Saito (1971)	−	Yamanaka *et al.* (1979)		
Pentachloroethane	76-01-7	202.3	−/−	+/+	Matsuoka *et al.* (1996)	−	Haworth *et al.* (1983)	+	McGregor *et al.* (1988b)
Tetrachloroethane	630-20-6	167.8	−/−	+/+	Matsuoka *et al.* (1996)	−	Haworth *et al.* (1983)	−, +	Myhr and Caspary (1991), McGregor *et al.* (1988b)
Urethane	51-79-6	89.1	+[a]	−/	Ishidate (1987)	−	McCann *et al.* (1975)	−	Amacher and Turner (1982)
Mitomycin C (MMC) IP	50-07-7	334.4	+/	−/	Ishidate (1987)	−,+[b]	Ishidate (1991)	+	Oberly *et al.* (1993)

CA, chromosome aberration assay; BRM, bacterial reverse mutation assay; MCGM, mammalian cell gene mutation assay; +, positive; +L, only positive on long-term treatment (48 h) in the CA; −, negative; IP, internal positive control.

[a]Positive response obtained at 8 mg/ml.

[b]Positive responses obtained in TA92 and TA94 strains.

Table III. Chemicals tested in the phase 2 study and previously published *in vitro* genotoxicity data

Chemical name	CAS no.	Mol. wt	CA			BRM		MCGM	
			Structural −S9/+S9	Numerical −S9/+S9	Reference		Reference		References
N-Aminoethyl ethanolamine	111-41-1	104.2	−/−	+L/−	Unpublished data	−	Leung (1994)	−	Leung (1994)
Benzyl acetate	110-11-4	150.2	−/−	−/−	Matsuoka *et al.* (1996)	−	Mortelmans *et al.* (1986)	+	McGregor *et al.* (1988b)
Bisphenol A	80-05-07	228.0	−/−		Ivett *et al.* (1989)	−	Haworth *et al.* (1983)	−	Myhr and Caspary (1991)
p-t-Butylphenol	98-54-5	150.2	−/+	+/−	Unpublished data	−	Unpublished data		
Cinnamyl anthranilate	87-29-7	253.0	−/−	−/−	Matsuoka *et al.* (1996)	−	Zeiger *et al.* (1988)	+	Myhr and Caspary (1991)
Colchicine	64-86-8	399.4	+/−	+/−	Galloway *et al.* (1987) Ando *et al.* (1995)	−	Mortelmans *et al.* (1986)	−	Clive *et al.* (1985)
2′-Deoxycoformycin	53910-25-1	268.3	+L/−	−/−	Otsuka *et al.* (1991)	+	Otsuka *et al.* (1991)		
1,3-Dimethylxanthine	58-55-9	180.2	+/−	−/−	Ishidate (1987)	−	Slamenova *et al.* (1986a)	+	Slamenova *et al.* (1986b)
Ethenzamide	938-73-8	165.2	+L/+	+L/−	Ishidate (1987)	−	Ashby and Paton (1993)		
Methacrylic acid 2-hydroxypropyl ester	923-26-2	144.2	+/+	+/+	Unpublished data	−	Unpublished data		
α-Naphthoquinoline	230-27-3	179.2	+/−	−/−	Ishidate (1987)	−	Hashimoto *et al.* (1979)		
Noscapine (1-narcotine)	128-62-1	413.4	+L/	+/	Ishidate (1987)	−	Bartsch *et al.* (1980)		
Oxytetracycline-HCl	2058-46-0	496.9	−/−		Anderson *et al.* (1990)	−	Mortelmans *et al.* (1986)	+	Myhr *et al.* (1990), McGregor *et al.* (1991)
Phenacetin	62-44-2	179.2	−/+	−/−	Ishidate (1987)	−, +[a]	Dunkel *et al.* (1985), Weinstein *et al.* (1981)	+	Caspary *et al.* (1988)
Phenylbutazone	50-33-9	308.4	+L/	−/−	Ishidate (1987)	−	Mortelmans *et al.* (1986)		
Thiabendazole	148-79-8	201.3	−/	+L/	Ishidate (1987)	−, +[a]	Zeiger *et al.* (1988)		
Triamterene	396-01-0	253.3	+/−	−/−	Ishidate (1987)	−	Mortelmans *et al.* (1986)		
Trichloroethylene	79-01-6	131.4	−/−	−/−	Matsuoka *et al.* (1996)	−	Waskell (1978)	+	Myhr and Caspary (1991)
Vinblastine sulfate	865-21-4	811.1	+/	+/	Segawa *et al.* (1979)	−	Heddle and Bruce (1977)	−	Suter *et al.* (1980)
Zearalenone	17924-92-4	318.4	+/+	+/−	Galloway *et al.* (1987)	−	Wehner *et al.* (1978a)	−	McGregor *et al.* (1988b)
Bromodichloromethane[b]									
Isophorone[b]									
Tetrachloroethane[b]									

CA, chromosome aberration assay; BRM, bacterial reverse mutation assay; MCGM, mammalian cell gene mutation assay; +, positive; +L, only positive on long-term treatment (48 h) in the CA; −, negative.

[a]Positive with hamster S9 but negative with rat S9.

[b]Also tested in the phase 1 study.

Each viability culture was then dispensed at 0.2 ml/well on 96-well plates (two plates for solvent control and one plate each for test chemical treatment and positive control). The plates were incubated at 37°C in a humidified incubator gassed with 5% CO_2 in air for 11–13 days.

Plating for 5-trifluorothymidine (TFT) resistance. After all the dilutions were prepared, TFT was added to all mutation assay cultures at a final concentration of 3 μg/ml. Each TFT-treated culture was dispensed at 0.2 ml/well on 96-well plates using a multichannel pipette (four plates for solvent control and two plates each for test chemical treatment and positive control). The plates were incubated for 12 days at 37°C in a humidified incubator gassed with 5% CO_2 in air to allow colony development.

Colony counting. The number of wells containing colonies was counted by naked eye or with the aid of a microscope. A well without colonies was classified as negative. The number of negative wells per plate was quantified for the survival (PE_0), viability (PE_2) and mutation (TFT) plates. For the TFT plates, we characterized colony size and morphology to obtain information about the mechanism of action of the test chemical. The colonies were characterized as follows:

(i) size: small, $\leqslant\frac{1}{4}$ of well diameter;
large, $>\frac{1}{4}$ of well diameter;
(ii) morphology: small, compact;
large, totally or partially diffuse.

Calculations

Survival and viability. From the zero term of the Poisson distribution, the probable number of clones/well (P) on microwell plates is

$$P = -\ln(EW/TW)$$

where EW is empty wells and TW is total wells (Furth *et al*., 1981).

The plating efficiency (PE) in any given culture is

$$PE = P/(\text{cells plated/well})$$

When 1.6 cells/well are plated on average for all survival and viability plates

$$PE = P/1.6.$$

The relative survival (RS) in each test culture will therefore be determined by comparing plating efficiencies in test and control cultures

$$RS\ (\%) = (PE_{test}/PE_{control})\times 100$$

Mutation frequency. Mutation frequency (MF) expressed as mutants/10^6 viable cells is calculated as

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

From the formula for PE and with the knowledge that 2×10^3 cells were plated/well for mutation to TFT resistance

$$PE_{mutant} = P_{mutant}/(2\times10^3)$$

$$PE_{viable} = P_{viable}/1.6$$

Relative total growth (RTG). Relative total growth (RTG) was calculated for estimating test chemical cytotoxicity. The relative suspension growth (RSG) was first calculated by daily cell growth (DCG)

$$RSG = [(DCG_1\times DCG_2)_{test}]/[(DCG_1\times DCG_2)_{control}]$$

DCG is the growth rate between days 0 and 1 (DCG_1) or between days 1 and 2 (DCG_2). The relative total growth (RTG) is calculated as

$$RTG\ (\%) = RSG\times RV\ (\%)$$

RV (relative viability) is calculated by comparing plating efficiencies in the test and control cultures at day 2.

Criteria of acceptable conditions

To demonstrate acceptable cell growth and maintenance throughout the experiment, the absolute plating efficiency for solvent control should be 60–140% for survival (PE_0) and 70–130% for viability (PE_2) according to the 1994 consensus agreement formed by the MLA workshop at Portland, OR (Clive *et al*., 1995). In the present studies, however, we accepted absolute plating efficiencies that were slightly outside these ranges. The experiments having extremely high (>170%) or low (<40%) plating efficiencies for solvent control were excluded from the evaluation. Spontaneous mutation frequencies $<60\times10^{-6}$ were noted but included.

When the mutation frequency of the positive control (MMS) in each experiment increased 2 times or more than that in the concurrent negative control, an experiment was accepted as having sufficient sensitivity.

In order to assess the mutagenic potential of a test chemical properly, a range of cytotoxicities should be tested with awareness that excessive cytotoxicity can cause false positive responses. According to the Portland agreement (Clive *et al*., 1995), the top concentration should show 10–20% RS or RTG, whichever is lower. In the present study, the data obtained under an excessive cytotoxic condition (<10% RS or RTG) were excluded from the evaluation.

Statistics

In the phase 1 study, we analyzed all results using a statistical package (Mutant™; UKEMS, York, UK) in accordance with the UKEMS guidelines (Robinson *et al*., 1989). This includes two procedures; one is pair-wise comparison of each treatment with the concurrent negative control and the other is testing for a linear trend between mutation frequency and concentration. Because most of the experiments in the phase 2 study were conducted with single cultures, the UKEMS statistics package was not applicable. These data were analyzed by a newly developed procedure with adjustment of the family-wise type I error (Hayashi *et al*., in preparation). The procedure consists of elimination of data showing a downturn phenomenon using the Simpson–Margolin procedure, dose–response effect evaluation and multiple comparisons with the concurrent control by a modified Dunnett's procedure.

Criteria for judgement

In the phase 1 study, we followed the statistical package analysis. When there were statistically significant responses ($P < 0.05$) in both the pair-wise comparison (at at least one concentration within the acceptable range) and the linear trend test, the experiment was designated +. When the pair-wise comparison or linear trend test was significant, it was designated P or L, respectively. When no significant response was obtained in either procedure, it was designated –. When the experiments contained extremely high (>170%) or low (<40%) PE_0 and PE_2 in the solvent control, the experiments were excluded from evaluation (UA). Experiments including data only at doses causing high cytotoxicity (<10% RS or RTG) were also excluded from evaluation (UE). ic signifies that negative results were obtained at the top concentration showing >20% RS and RTG and nc signifies that negative results were obtained at concentrations up to 5 mg/ml showing >20% RS and RTG.

In the phase 2 study, following a newly developed statistical procedure for single culture assay, we used multiple comparison with the concurrent control and dose–response effect evaluation eliminating data showing a downturn phenomenon (the procedure will be reported). We judged responses as positive (+) if both steps were significant and as negative (–) if either step was not significant. Experiments that were left with fewer than three treatment doses due to exclusion of the doses showing <10% RS or RTG or a downturn phenomenon were judged to be unavailable for evaluation (UE). UA, ic and nc have the same meaning as in the phase 1 study.

For the final judgment, each chemical was ranked as + (positive), – (negative), I (inconclusive) or E (equivocal) in consideration of the concordance between laboratories or between the phase 1 and 2 studies. Basically, a test chemical evaluated to be + or – by both laboratories was judged as 'positive' or 'negative', respectively, and chemicals with discordant results between laboratories were judged as 'inconclusive'. When a confirmation test by two laboratories was performed because of an inconclusive call in the initial study and it again showed discordant results between laboratories, this chemical was judged as 'equivocal'. Other cases were evaluated individually.

Results and discussion

Comparison of experimental conditions and data qualities

In the phase 1 study, 47 of the 49 participating laboratories provided a final report. Sixteen chemicals were tested by two laboratories and five were tested by three laboratories. A total of 94 experiments were carried out (47 laboratories tested one chemical with and without S9 mix). In the phase 2 study, 45 reports were provided to the organizing committee. Twenty two chemicals were tested by two laboratories and one chemical was tested by one laboratory. In total, 89 experiments were performed (one laboratory tested a chemical in the presence of S9 mix only). The experimental condition and acceptability of the data are summarized in Tables IV and V.

Choice of solvent. Although all test chemicals were coded and the choice of solvent was the responsibility of each laboratory, the coincidence of choice of solvent for each test chemical was 81% (17/21) in the first and 77% (17/22) in the second

Table IV. Comparison of experimental conditions and acceptability of data in the phase 1 study

Chemical name	Choice of solvent[a]			Correspondence of testing doses between labs[b] (−S9/+S9)	plating efficiency of solvent control						Cytotoxicity within 10−20%[c]						Data with higher heterogeneity (−S9/+S9)
					PE_0(–S9/+S9)			PE_2(–S9/+S9)			%RS (–S9/+S9)			%RS (–S9/+S9)			
	A	B	C		A	B	C	A	B	C	A	B	C	A	B	C	A B C
Arsenic trioxide	RPMI (p)	RPMI		2/2	134/114	98/97		105/121	126/103		20/35	17/34		4−24/12	16/33		
Benzene	DMSO	DMSO (s)		2/2	119/100	19/59		115/129	95/77		21/13	36/15		9−60/9−51	15/15		/2
Bromodichloromethane	DMSO	DMSO		2/1	93/83	102/77		146/114	100/84		0−54/11	7−74/34		0−31/6−29	3−67/52		
Cadmium sulphate	Saline	Saline		2/2	66/66	87/87		79/130	106/77		1−32/6−100	11/15		0−37/3−78	8−100/0−22		
Chlorendic acid	DMSO	DMSO		2/2	62/86	135/114		114/114	106/87		87/14	34/13		87/8−22	30/10		
Chlorodibromomethane	DMSO	DMSO		2/1	63/23	94/82		70/85	75/73		2−86/10	60/23		0−42/24	49/14		
Cytosine arabinoside	RPMI	RPMI	RPMI	2/2	87/74	207/69	97/97	127/129	74/82	106/106	11−16/24	31/37	26/24	10−18/20	43/23	14/12−19	/1
Dideoxycytidine (DDC)	RPMI	RPMI		2/2	73/81	91/65		93/88	71/72		87[e]/95[e]	88[e]/71[e]		84[e]/78[e]	91[e]/71[e]		
Diethylstilbestrol (DES)	DMSO (p)	DMSO (p)	DMSO	2/2	135/65	103/85	105/110	91/102	127/116	109/104	79/26	1−67/13	10/16	100/17	0−53/9−43	13/10−12	
Eugenol	DMSO	DMSO		2/2	79/71	89/66		102/98	89/117		13/18	87/8−34		12/15	54/7−26		2/
5-Fluorouracil (5-FU)	RPMI	RPMI		2/2	140/111	93/83		105/105	88/112		9−35/6−40	4−27/27		1−25/2−45	4−100/0−24		
Griseofulvin	RPMI (p)	RPMI (p)	DMSO (p)	1/2	62/114	159/71	84/85	73/85	117/131	83/81	70/60	69/90	79/85	0−28/2−38	0−100/26	0−47/9−52	
Hexamethyl phosphoramide	DMSO	RPMI		2/0	69/85	87/77		86/94	94/98		32[e]/41	34[e]/85[e]		28[e]/54	25[e]/61[e]		
Hydroxyurea	RPMI	RPMI		2/2	106/116	91/99		114/97	86/85		32/21	25/18		23/15−17	18/29−35		
Isophorone	DMSO	DMSO		2/2	90/62	76/97		89/69	114/94		10/6	84/24		8−37/3−26	73/14		1/
Methotrexate (MTX)	DMSO	RPMI		2/2	131/179	79/70		110/94	109/100		14/10	7−20/14		10/4−100	16/13		
Monocrotaline	DMSO	DMSO		1/2	74/67	102/72		85/91	106/71		20/12	18/16		11/4−29	18/16−18		
Pentachloroethane	DMSO	DMSO		2/2	112/67	93/73		108/92	88/97		14/0−86	24/100		5−39/0−93	44/67		
Tetrachloroethane	DMSO	DMSO		2/1	56/60	77/106		60/80	78/76		9−32/44	60/63		11/35	59/29		
Urethane	RPMI	RPMI	DW	2/2	62/96	47/64	133/123	109/105	74/87	98/134	24[e]/77[e]	77[e]/99[e]	46[e]/100[e]	22[e]/76[e]	90[e]/100[e]	83[e]/100[e]	
Mitomycin C IP	RPMI	DMSO	DMSO	2/2	91/63	120/125	76/72	88/62	70/88	69/84	4−31/12	5−28/4 0	15/8−38	15/8−57	15/32	11/17	/1

[a](p) and (s) indicate precipitation and separation, respectively, at at least one concentration.
[b]2, two or more doses overlap between laboratories; 1, one dose overlap; 0, no dose overlap.
[c]RS or RTG within 10−20% or the lowest RS or RTG are shown. 4−24 means that the lowest RS or RTG is 4% and the next lowest RS or RTG is 24%.
[d]The number of concentrations showing a higher heterogeneity factor.
[e]The compounds were not highly cytotoxic, even at the limit dose (5 mg/ml).
IP, internal positive control.

Table V. Comparison of experimental conditions and acceptability of data in the phase 2 study

Chemical name	Choice of solvent[a]		Correspondence of testing doses between labs[b] (−S9/+S0)	Plating efficiency of solvent control				Cytotoxicity within 10−20%[c]			
				PE_0 (−S9/+S9)		PE_2 (−S9/+S9)		% RS (−S9/+S9)		% RTG (−S9/+S9)	
	A	B		A	B	A	B	A	B	A	B
N-Aminoethyl ethanolamine	RPMI	RPMI	2/2	71/60	58/57	91/110	26/38	12/4−54	6−73/13	13/3−40	0−74/0−42
Benzyl acetate	DMSO	DMSO	2/2	67/76	106/138	130/141	94/120	14/32	0−79/3−74	21/26	0−31/1−81
Bisphenol A	DMSO	DMSO	2/2	91/77	92/89	118/106	95/89	0−96/0−89	14/9−30	0−48/0−32	10−16/14
p-t-Butylphenol	DMSO	DMSO	2/2	77/75	51/59	70/45	85/85	33/9−80	20/8−27	42/6−56	19/16
Cinnamyl anthranilate	DMSO	DMSO	2/2	110/79	75/78	94/83	102/93	15/4−23	3−46/9−75	10/12	1−31/3−67
Colchicine	RPMI	Saline	2/2	92/84	82/92	94/88	127/98	8−25/10	19/13	1−30/16	16/4−22
2′-Deoxycoformycin	Saline	RPMI	2/2	88/62	82/36	105/105	127/118	80[e]/73[e]	71[e]/85[e]	57[e]/82[e]	53[e]/73[e]
1,3-Dimethylxanthine	RPMI			105/108		100/89		85[e]/71[e]		70[e]/93[e]	
Ethenzamide	RPMI (p)	RPMI (p)	2/2	76/101	64/63	95/112	77/71	50/26	44[e]/13−14	14/19	35[e]/7−30
Methacrylic acid 2-hydroxypropyl ester	RPMI	DMSO	2/2	103/87	84/60	101/97	61/74	27/17	30/17	6−21/13	8−63/3−25
α-Naphthoquinoline	DMSO	DMSO	2/2	92/98	104/86	89/92	105/96	7−65/13	48/68	15−17/16	17/51
Noscapine (1-narcotine)	DMSO (p)	RPMI (p)	0/0	108/98	104/78	120/82	103/92	69/80	66[e]/66[e]	0−40/1−53	29[e]/29[e]
Oxytetracycline-HCl	Saline	DMSO	2/2	92/88	116/101	101/88	116/100	8−34/14	14/16	19/14−15	12/0−24
Phenacetin	DMSO (p)	DMSO (p)	2/2	65/91	84/100	118/71	81/106	100/31	100/37	76/15	14/0−22
Phenylbutazone	DMSO	DMSO	2/2	80/98	83/80	85/83	133/116	13/2−52	18/14	7−53/11	0−35/0−34
Thiabendazole	DMSO (p)	DMSO	2/2	118/88	127/70	127/114	106/114	76/70	60/50	13/18	1−90/0−80
Triamterene	DMSO	DMSO	2/2	100/105	85/84	103/103	96/75	19/7−58	9−21/8−31	11/1−74	13/7−32
Trichloroethylene	DMSO	DMSO	1/1	88/98	101/81	83/108	100/116	0−80/15	0−43/0−23	0−35/13	0−53/0−20
Vinblastine sulfate	DMSO	Saline	2/2	70/108	127/130	105/81	77/110	99/45	91/96	19/5−38	3−93/0−23
Zearalenone	DMSO	Saline	2/2	100/116	112/79	88/87	145/108	14/17	0−55/7−41	7−23/7−25	0−54/2−28
Bromodichloromethane[f]	DMSO	DMSO	2/2	71/98	105/76	78/97	98/101	0−87/11	0−47/26	0−50/4−28	0−33/0−23
Isophorone[f]	DMSO	DMSO	2/2	61/95	95/76	94/97	89/83	10−14/10	13−14/10	9−41/14−17	19/10−19
Tetrachloroethane[f]	DMSO	DMSO	/2	71/51	/60	84/103	/91	0−100/71	/11	0−100/14	/14

[a](p) and (s) indicate precipitation and separation, respectively, at at least one concentration.
[b]2, two or more doses overlap between laboratories; 1, one dose overlap; 0, no dose overlap.
[c]RS or RTG within 10−20% or the lowest RS or RTG are shown. 4−54 means that the lowest RS or RTG is 4% and the next lowest RS or RTG is 54%.
[d]The number of concentrations showing a higher heterogeneity factor.
[e]The compound was not highly cytotoxic, even at the limit dose (5 mg/ml).
[f]Also tested in the phase 1 study.

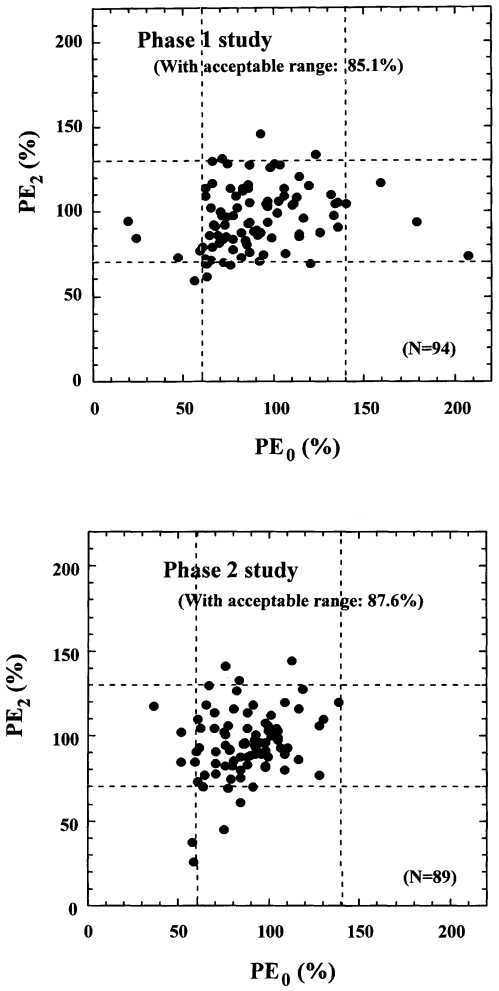

Fig. 1. The distribution of PE_0 and PE_2 solvent control values in the phase 1 (upper) and phase 2 (lower) studies. Broken lines indicate PE_0 of 60 and 140% and PE_2 of 70 and 130%.

study, indicating that each laboratory performed the solubility tests carefully and accurately.

Four chemicals were tested in the presence of precipitates or separation by at least one laboratory. Some discrepancies in the observation of the precipitates between laboratories using the same solvents might have been due to differences in concentrations tested or to record-keeping omissions (arsenic trioxide, benzene and diethylstilbestrol in the phase 1, thiabendazole in the phase 2 and tetrachloroethane in both studies).

Dose finding. Almost all chemicals were tested at similar dose ranges in the presence and absence of S9 mix in the different laboratories. A few exceptions showed remarkable dose range discrepancies (hexamethyl phosphoramide with S9 mix in the phase 1 and noscapine with and without S9 mix in the phase 2 study). These discrepancies may have been due to the different choice of solvent (RPMI versus DMSO).

Plating efficiencies of solvent control. As criteria for acceptance of the MLA data, we first defined that the absolute plating efficiency for the solvent control should be 60–140% for survival (PE_0) and 70–130% for viability (PE_2). In the 94 experiments of the phase 1 study, 87 (93%) and 88 (94%) solvent control values, respectively, were within the the acceptable range for PE_0 and PE_2 (Fig. 1). In the 89 experiments of the phase 2 study, 83 (93%) and 82 (93%) solvent control values, respectively, were within the acceptable range for PE_0

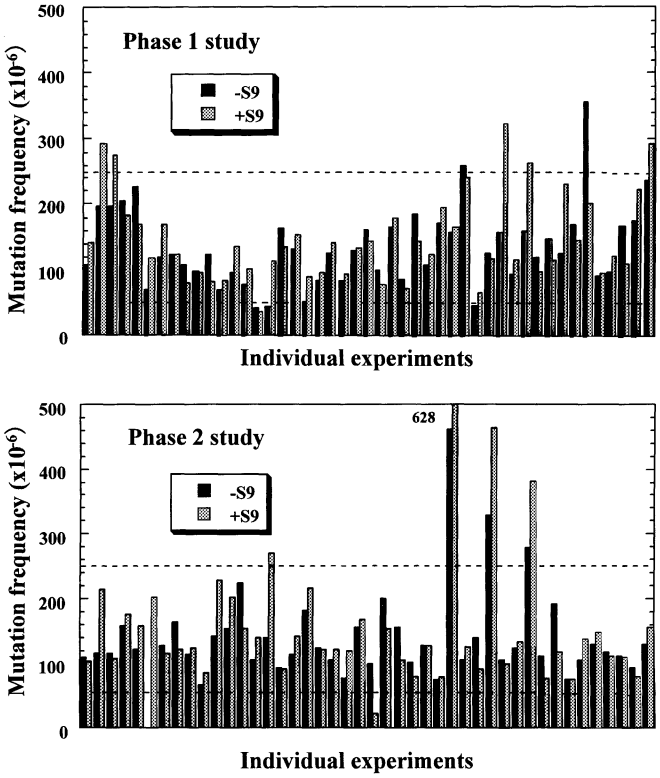

Fig. 2. The distribution of spontaneous mutation frequencies in the phase 1 (upper) and phase 2 (lower) studies. Broken lines indicate mutation frequencies of 50×10^{-6} and 250×10^{-6}.

and PE_2 (Fig. 1). The experiments having both acceptable PE_0 and PE_2 in the phase 1 and 2 studies were 80 (85%) and 78 (88%), respectively. This indicates that almost all laboratories maintained the cell cultures and performed cell counts properly.

Spontaneous mutation frequency. The distribution of spontaneous mutation frequencies for all experiments are shown in Figure 2. In 88% of the phase 1 and 91% of the phase 2 experiments spontaneous mutation frequencies were within the range $50–250\times10^{-6}$. The 1994 consensus agreed that spontaneous mutation frequencies $<60\times10^{-6}$ should be viewed with caution for the microwell method, but no statement was made about an acceptable upper limit (Clive *et al.*, 1995).

Mutation frequency of positive control. MMS, the positive control chemical in the tests without S9 mix, showed clear increases in mutation frequencies in all experiments, except for one experiment (phase 2, *p*-t-butylphenol, –S9, lab B). In this experiment the mutation frequency of MMS was less than twice the concurrent solvent control value and the data was excluded from result evaluation. All experiments with S9 mix obtained >2-fold increases in mutation frequencies by the positive control chemical, CP. These results indicate sufficient sensitivity of almost all experiments in this collaborative study.

Top concentration. According to the 1994 consensus agreement (Clive *et al.*, 1995), the highest concentration should cause 10–20% RS or RTG. In the 94 phase 1 experiments, 13 experiments on three chemicals (DDC, hexamethyl phosphoramide and urethane) did not show such severe cytotoxicity even at the limit concentration (5 mg/ml). Only 29 (36%) of the remaining 81 experiments fell into this narrow range of both RS and RTG, although many of them were tested up to severe cytotoxic doses. Thirty nine (48%) experiments did not test concentrations causing 10–20% RS or RTG.

Table VI. Results of the phase 1 MLA study

Chemical	Results (−S9/+S9)[a]			Judgement
	lab A	lab B	lab C	
Arsenic trioxide	+/P	+/+		Positive (+/+)
Benzene	−/L	UA/+		Positive (−/+)
Bromodichloromethane	−/+	−/−(ic)		Inconclusive (−/I)
Cadmium sulphate	+/+	UE/+		Positive (+/+)
Chlorendic acid	+/−	−(ic)/+		Inconclusive (I/I)
Chlorodibromomethane	−/UA	−(ic)/+		Positive (−/+)
Cytosine arabinoside (Ara C)	+/+	UA/+	+/+	Positive (+/+)
Dideoxycytidine (DDC)	−(nc)/−(nc)	−(nc)/−(nc)		Negative (−/−)
Diethylstilbestrol (DES)	−(ic)/+	−/+	−/+	Positive (−/+)
Eugenol	+/+	+/+		Positive (+/+)
5-Florouracil (5-FU)	+/+	UE/+		Positive (+/+)
Griseofulvin	+/−	+/P	+/−	Positive (+/−)
Hexamethyl phosphoramide	+/L	+/+		Positive (+/+)
Hydroxyurea	+/+	+/+		Positive (+/+)
Isophorone	−/+	−(ic)/−		Inconclusive (−/I)
Methotrexate (MTX)	+/UA	+/+		Positive (+/+)
Monocrotaline	+/+	+/+		Positive (+/+)
Pentachloroethane	+/−	+/−(ic)		Positive (+/−)
Tetrachloroethane	−/−(ic)	−(ic)/P		Inconclusive (−/I)
Urethane	+/−(nc)	−(nc)/−(nc)	−(nc)/−(nc)	Negative (−/−)
Mitomycin C, IP	+/+	+/+	+/+	Positive (+/+)

[a]Results obtained without/with S9 mix are shown for each laboratory.
+, positive response, statistical significance in both pair-wise comparison and linear trend test; −, negative response, no statistical significance in either analysis; P, statistical significance was obtained only in pair-wise comparison; L, statistical significance was obtained only in linear trend test; UA, unacceptable data with extremely high (>170%) or low (<40%) PE_0 or PE_2 in solvent control; UE, unavailable for evaluation due to exclusion of the data accompanied by high cytotoxicity (<10% RS or RTG); I, inconclusive because of discrepant results between two laboratories; ic, negative result obtained at the top concentration with >20% RS and RTG; nc, negative result obtained at concentrations up to the limit dose (5 mg/ml) with >20% RS and RTG; IP, internal positive control.

In the 89 experiments of the phase 2 study, nine dealing with four chemicals (2′-deoxycoformycin, 1,3-dimethylxanthine, ethenzamide and noscapine) did not yield severe cytotoxicity even at the limit concentration. Most of the remaining 80 experiments showed clear cytotoxicity, but only 29 (36%) and 33 (41%) reached the recommended RS and RTG, respectively. Thirty four (43%) experiments did not test concentrations causing 10–20% RS or RTG.

Thus, about half of the experiments fell short of the recommended top concentration, but most of them were not far off. The results of the preliminary cytotoxicity tests coincided well with those of the main experiments, indicating that dose finding was performed accurately. The results of the present study suggest that it is important to test up to <20% RS or RTG rather than settle for 10–20% RS or RTG.

Heterogeneity. The UKEMS guidelines and the 1994 consensus agreement (Clive *et al.*, 1995) recommend the use of duplicate cultures, especially when the data are processed with the statistical package Mutant™ for the microwell method. In the phase 1 study, the data from duplicate cultures corresponded well and few (5/94 or 5%) showed appreciable heterogeneity under the present conditions applied. This indicates that the microwell method under the present protocol did not result in serious discordance. We therefore used single cultures in the phase 2 study, as a rule.

Evaluation of mutagenicity of test chemicals

Phase 1 study (20 chemicals and an internal positive control, MMC). The mutagenicity of each chemical is given in Table VI; the results of all experiments and the mutation frequency caused by each chemical are shown in Appendix 1.

Arsenic trioxide: positive (–S9/+S9: +/+). Arsenic trioxide exhibited statistically significant positive responses with and without S9 mix in both laboratories. The maximum induced mutation frequencies were <3 times the spontaneous one. The chemical was insoluble at high concentration (>5 μg/ml). While cytotoxicity (RS) after treatment with precipitates was not as strong, severely decreased RTG (0% at 10 μg/ml) was seen. This delayed cytotoxicity may have been caused by continuous exposure to precipitates during the expression time. In laboratory A (lab A), a decrease in mutation induction at the highest concentration might have been associated with this delayed cytotoxicity. It was difficult to obtain 10–20% RS at the top concentration for this chemical.

Benzene: positive (–S9/+S9: –/+). In lab A, benzene showed weak positive responses in the presence of S9 mix but not in its absence. Experiments in laboratory B (lab B) showed extremely low PE_0 in the solvent control without S9 mix and we excluded these data from evaluation. Although heterogeneity factors of high statistical significance were seen in the PE_2 data in the presence of S9 mix in lab B, the response was dose-dependent and reproducible in both laboratories. We judged benzene as a positive chemical in the presence of S9 mix, which is inconsistent with results obtained by the agar method (Oberly *et al.*, 1984).

Bromodichloromethane: inconclusive (–S9/+S9: –/I). The cytotoxic response to bromodichloromethane was higher in the presence of S9 mix than in its absence. A positive response was obtained with S9 mix in lab A but not in lab B. The positive response was statistically significant in both pair-wise comparison and linear trend tests, but the maximum mutation frequency was <2 times the spontaneous one. On the other hand, negative results were obtained in lab B with S9 mix

under the same cytotoxic conditions (34% RS) as in lab A, though RS and RTG did not reach 20%. We called bromodichloromethane inconclusive, but McGregor *et al*. (1988a) demonstrated that it evoked a clear positive response in the MLA in the presence of S9 mix.

Cadmium sulfate: positive (–S9/+S9: +/+). Cadmium sulfate caused a clear positive response in all experiments except for one in lab B without S9 mix. All data obtained without S9 mix in lab B were generated under strong cytotoxic conditions (<10% RTG) and were not used in the evaluation. Cytotoxicity of this chemical increased steeply over 0.25–0.50 μg/ml.

Chlorendic acid: inconclusive (–S9/+S9: I/I). Chlorendic acid caused positive responses without S9 mix in lab A and with S9 mix in lab B. The lab A result was obtained under weak cytotoxic conditions (87% RS and RTG), but the lab B experiments did not show any positive responses under the more cytotoxic conditions (34% RS and 30% RTG). S9 mix increased cytotoxicity slightly but hardly affected mutagenicity. We judged this chemical to be inconclusive because of the discordant results between two laboratories. A positive MLA result for chlorendic acid was previously reported without S9 mix (McGregor *et al*., 1988a) and the authors suggested that this chemical exhibited a mutagenic response only within a narrow range of a highly toxic dose level.

Chlorodibromomethane: positive (–S9/+S9: –/+). S9 mix lowered the concentration at which chlorodibromomethane was cytotoxic. In the presence of S9 mix positive responses were obtained in lab B, but the data from lab A indicating positive responses were excluded from evaluation because of an unacceptable PE_0 value (23%) in the solvent control. In the absence of S9 mix, there were no significant mutagenic response in either laboratory, although a positive response was seen in lab A at the top concentration, which caused severe cytotoxicity (2% RS). We judged chlorodibromomethane to be positive with S9 mix. McGregor *et al*. (1991) demonstrated that this chemical showed mutagenicity in the absence of S9 mix in the MLA under highly cytotoxic conditions.

Cytosine arabinoside (Ara C): positive (–S9/+S9: +/+). Ara C was tested by three laboratories and caused clear positive responses in all experiments, though the PE_0 in lab B was unacceptable in the absence of S9 mix. The addition of S9 mix reduced cytotoxicity and mutagenicity. The mutagenic activity was related to the degree of cytotoxicity achieved and even at low toxic concentrations (80–90% RS), mutants were induced. Ara C is one of the potent mutagens in the MLA. The mutagenicity of cytidine analogs including Ara C in the MLA was previously reported by McGregor *et al*. (1989). They and Dickins *et al*. (1985) demonstrated that cytidine analogs induce TFT-resistant mutants, but not 6-thioguanine-resistant mutants, implying that they are clastogenic rather than mutagenic. An increase in small colonies, however, was not clear in this study.

Dideoxycytidine (DDC): negative (–S9/+S9: –/–). DDC up to 5 mg/ml caused neither enough cytotoxicity nor any mutagenicity with and without S9 mix in both laboratories. In a CA with cultured human lymphocytes DDC caused a positive response following long continuous treatment without S9 mix (Chetelat, 1987). It is possible that continuous treatment could also cause a positive response in the MLA.

Diethylstilbestrol (DES): positive (–S9/+S9: –/+). DES was tested by three laboratories. It caused positive responses in three experiments only in the presence of S9 mix, indicating that DES may need metabolic activation to express its mutagenicity. The maximum mutation frequencies were <3 times the spontaneous one and were statistically significant. It is interesting that cytotoxic responses were similar in the presence and absence of S9 mix. Myhr and Caspary (1988) also observed DES mutagenicity in the MLA only in the presence of S9 mix and demonstrated that the S9 mix did not metabolically activate DES. They speculated that the S9 protein–lipid complex altered the effective concentration of DES by influencing its solubility and/or cellular uptake.

Eugenol: positive (–S9/+S9: +/+). Eugenol caused clear dose-dependent responses in both laboratories with and without S9 mix. Cytotoxicity and mutagenicity increased in the presence of S9 mix.

5-Fluorouracil (5FU): positive (–S9/+S9: +/+). S9 mix had no effect on the cytotoxicity or mutagenicity of 5FU and the responses were almost dose-dependent. The RTG was generally lower than the RS and PE_2 did not recover well, suggesting strong delayed cytotoxicity. Lab B's experiment without S9 mix was excluded from evaluation because all treatments caused severe cytotoxicity (<10% RTG).

Griseofulvin: positive (–S9/+S9: +/–). Griseofulvin was difficult to test because of its poor solubility. The cells were necessarily exposed to precipitated chemical during the expression as well as treatment times, because the precipitated chemical was impossible to remove completely after treatment. This chemical did not show severe cytotoxicity (RS) even at the highest concentration. Continuous exposure of the precipitated chemical during the expression time, however, brought strong cytotoxicity, resulting in quite low RTG and PE_2 values. It was therefore impossible to measure mutation frequencies at >200 μg/ml in spite of the weak cytotoxicity indicated by RS. RS might be an unsuitable cytotoxic endpoint for this kind of chemical. Indeed, all three laboratories failed to find appropriate doses in the preliminary dose-finding tests on the basis of RS.

Low dose mutation frequencies were considered to be reliable. Positive responses were obtained in three laboratories for concentrations between 50 and 220 μg/ml, in which 80–90% RS was seen with and without S9 mix. The responses were weak (2–4 times background), but statistically significant. Griseofulvin was judged positive in the absence of S9 mix because its effect was reproducible.

Hexamethyl phosphoramide: positive (–S9/+S9: +/+). In the absence of S9 mix, hexamethyl phosphoramide showed cytotoxicity at 5 mg/ml, at which the RS values were 32 and 34% in lab A and lab B, respectively. Both laboratories obtained clear positive responses only at this limit concentration. In the presence of S9 mix, both laboratories obtained statistically significant responses. The cytotoxicities observed in the two laboratories were remarkably different, however, possibly because of the different choices of solvent (DMSO versus RPMI). Hexamethyl phosphoramide was judged to be positive in both the presence and absence of S9 mix.

Hydroxyurea: positive (–S9/+S9: +/+). Responses to hydroxyurea were significant in both pair-wise comparisons and linear trend tests for all experiments with and without S9 mix.

Isophorone: inconclusive (–S9/+S9: –/I). Lab A obtained a dose-dependent and statistically significant positive response for isophorone with S9 mix, but the maximum mutation frequency was <2 times the spontaneous one. Lab B obtained negative responses at similar doses. In the absence of S9 mix,

statistically significant positive responses were not obtained in either laboratory, although lab B did not test the chemical at cytotoxic enough conditions. Similar disparities are seen in the literature. McGregor *et al*. (1988b) reported that isophorone (1 mg/ml) showed clear positive responses in the presence of S9 mix, while O'Donoghue *et al*. (1988) did not obtain a positive response in similar experimental conditions.

Methotrexate (MTX): positive (–S9/+S9: +/+). MTX mutation frequencies increased dose-dependently with and without S9 mix. Statistically significant responses were obtained in all experiments except for the experiment in lab A with S9 mix. That experiment was excluded from evaluation, because it had an unacceptable PE_0 in the solvent control and all treatments exhibited extremely high cytotoxicity (<10% RS or RTG). Because MTX induces mutations in the MLA with the agar method, but not in an *hprt*–V79 assay system (Clive *et al*., 1979; Dickins *et al*., 1985), MTX may induce gross structural changes rather than small DNA changes, although an increase in small colonies, which are thought to have gross DNA changes, was not clear in this study.

Monocrotaline: positive (–S9/+S9: +/+). Monocrotaline caused clear dose-dependent responses in all experiments. The chemical was more mutagenic in the presence of S9 mix than in its absence, indicating that its mutagenicity was enhanced by metabolic activation. S9 mix also enhanced cytotoxicity.

Pentachloroethane: positive (–S9/+S9: +/–). Pentachloroethane mutagenicity was demonstrated without S9 mix in two laboratories, but the maximum mutation frequency was <3 times the spontaneous one. In the presence of S9 mix the chemical was negative, although the lab B experiments were not performed under sufficiently cytotoxic conditions. Pentachloroethane mutagenicity in the absence of S9 mix in the MLA was also observed by McGregor *et al*. (1988b).

Tetrachloroethane: inconclusive (–S9/+S9: –/I). Tetrachloroethane did not induce dose-dependent responses. Among all the experiments, only one dose in lab B with S9 mix showed a statistically significant response in a pair-wise comparison. The mutagenicity of tetrachloroethane in the MLA has been debated and discrepant results were obtained in two experiments by the NTP; McGregor *et al*. (1988b) reported a positive result in the presence and absence of S9 mix, while Myhr and Caspary (1991) reported negative results under both conditions.

Urethane: negative (–S9/+S9: –/–). Urethane was tested by three laboratories. All experiments, except for that of lab A without S9 mix, showed no cytotoxic and no mutagenic responses. The exceptional positive result was suspect, however, because cytotoxicity was observed at 2.5 mg/ml whereas urethane is normally not cytotoxic even at 5 mg/ml. In the CA, a positive response for urethane was observed only at extremely high doses (8 mg/ml) with 48 h continuous treatment (Ishidate, 1987).

Internal positive control, mitomycin C (MMC): positive (–S9/+S9: +/+). MMC, the internal positive control compound, was tested by three laboratories and all experiments showed clear positive responses, indicating that the overall quality of this collaborative study was adequate. Interestingly, MMC induced large colonies as well as small ones, although it is viewed as a model clastogen. MMC is less clastogenic than X-rays, bleomycins and actinomycin D according to studies comparing mutation induction at the heterozygous *gpt* locus in AS52 cells and the hemizygous *hprt* locus in CHO-K1-BH4 cells (Tindall and Stankowski, 1987; Honma *et al*., 1997).

Phase 2 study (23 chemicals). The mutagenicity of each chemical is given in Table VII; the results of all experiments and the mutation frequency caused by each chemical are shown in Appendix 2.

N-*Aminoethyl ethanolamine: positive (–S9/+S9: +/+).* A dose-dependent positive response was obtained for *N*-aminoethyl ethanolamine in lab A regardless of the presence of S9 mix. We excluded all lab B data because of unacceptable PE_0 values in the solvent control. A battery of *in vitro* and *in vivo* genotoxicity assays, including the CHO/*HPRT* gene mutation assay, showed that this chemical had no mutagenic potential (Leung, 1994) and might be a MLA unique positive chemical. Recently, however, the CA using CHL cells showed that this chemical induced numerical changes (polyploidy) but not structural changes (Tanaka *et al*., personal communication). This suggests that the MLA can detect chemicals that cause numerical changes, as well as structural changes, of chromosomes.

Benzyl acetate: inconclusive (–S9/+S9: –/I). Benzyl acetate was negative in the absence of S9 mix in both laboratories. In the presence of S9 mix, the lab A experiment exhibited a dose-dependent and statistically significant response, though the induced mutation frequency was <3 times the spontaneous one. Lab B failed to obtain positive responses using similar dose ranges. The mutagenicity of benzyl acetate in the CA is debatable (Galloway *et al*., 1987; Ishidate, 1987), although its CA-negative response was recently confirmed by Matsuoka *et al*. (1996). McGregor *et al*. (1988b) demonstrated a positive response for this chemical in the MLA without S9 mix.

Bisphenol A: inconclusive (–S9/+S9: I/I). Both with and without S9 mix, bisphenol A showed statistically significant responses in lab A but not in lab B. Therefore, this chemical was judged inconclusive with and without S9 mix. NTP reported that bisphenol A was non-mutagenic in the MLA (Ivett *et al*., 1989; Myhr and Caspary, 1991).

p-*t-Butylphenol: negative (–S9/+S9: –/–).* The experiment without S9 mix in lab B was unacceptable, because the mutation frequency of the positive control was <2 times that in the solvent control. No statistically significant responses were found in any experiment. We found no published genotoxic data for this chemical. According to a personal communication (Tanaka *et al*.), *p*-t-butylphenol was negative in the BRM but positive in the CA, in which it weakly induced structural changes with S9 mix and clear numerical changes without S9 mix.

Cinnamyl anthranilate: positive (–S9/+S9: +/I). Significant but small increases in mutation frequency were observed in the absence and presence of S9 mix in lab A. In lab B, the experiment without S9 mix was unavailable for evaluation (UE) because of an insufficient number of treatment doses (<3) resulting from elimination of a dose showing severe cytotoxicity. The result with S9 mix in lab B was negative. We judged this chemical to be positive in the absence of S9 mix. According to the review by Tennant *et al*. (1987), cinnamyl anthranilate examined in four widely used genotoxicity assays (BRM, CA, MLA and sister chromatid exchange) exhibited positive responses only in the MLA. Matsuoka *et al*. (1996) reported that the chemical did not yield any structural and numerical abnormalities in CHL cells in the CA. Cinnamyl anthranilate might be a MLA unique positive chemical.

Colchicine: negative (–S9/+S9: –/–). No statistically significant responses were observed in any experiments. It was difficult to test this chemical in the MLA because the delayed cytotoxicity brought about great differences between RS and

Table VII. Results of the phase 2 MLA study

Chemical	Results (−S9/+S9)[a]		Judgement
	lab A	lab B	
N-Aminoethyl ethanolamine	+/+	UA/UA	Positive (+/+)
Benzyl acetate	−/+	−/−	Inconclusive (−/I)
Bisphenol A	+/+	−/−	Inconclusive (I/I)
p-t-Butylphenol	−(ic)/−	UA/−	Negative (−/−)
Cinnamyl anthranilate	+/+	UE/−	Positive (+/I)
Colchicine	UE/−	−/−	Negative (−/−)
2′-Deoxycoformycin	−(nc)/−(nc)	−(nc)/UA	Negative (−/−)
1,3-Dimethylxanthine	−(nc)/−(nc)		Negative (−/−)
Ethenzamide	−/UE	+/+	Positive (I/+)
Methacrylic acid 2-hydroxypropyl ester	+/+	+/+	Positive (+/+)
α-Naphthoquinoline	+/+	−/+	Positive (I/+)
Noscapine (l-narcotine)	+/−	−(nc)/−(nc)	Inconclusive (I/−)
Oxytetracycline-HCl	−/−	+/+	Inconclusive (I/I)
Phenacetine	−(ic)/+	+/−	Inconclusive (I/I)
Phenylbutazone	−/+	+/+	Positive (I/+)
Thiabendazole	−/−	−/−	Negative (−/−)
Triamterene	+/−	+/UE	Positive (+/−)
Trichloroethylene	−/+	−/+	Positive (−/+)
Vinblastine sulfate	+/+	−/−	Inconclusive (I/I)
Zearalenone	−/UE	−/−	Negative (−/−)
Bromodichloromethane[b]	−/+	−/−	Equivocal (−/E)
Isophorone[b]	−/−	UE/UE	Negative (−/−)
Tetrachloroethane[b]	−/−	/−	Negative (−/−)

[a]Results obtained without/with S9 mix are shown for each laboratory.
[b]Also tested in the phase 1 study.
+, positive response, statistical significance in both dose−response effect evaluation and multiple comparison with concurrent control; −, negative response, no statistical significance in at least one of two analyses; UA, unacceptable data with extremely high (>170%) or low (<40%) PE_0 or PE_2 in solvent control or insufficient response of positive control (<2 times solvent control); UE, unavailable for evaluation because of insufficient number of treatment doses (<3) as a result of exclusion of highly cytotoxic data (<10% RS or RTG) or a downturn phenomenon; I, inconclusive because of discrepant results between two laboratories; E, equivocal because of marginal responses in both phase 1 and 2 studies; ic, negative result obtained at the top concentration with >20% RS and RTG; nc, negative result obtained at concentration up to the limit dose (5 mg/ml) with >20% RS and RTG.

RTG. Colchicine was reported to induce predominantly polyploidy in the CA (Galloway *et al*., 1987) and Ando *et al*. (1995) reported that it induces only polyploidy. Clive *et al*. (1985) also failed to demonstrate colchicine mutagenicity in the MLA, suggesting that the MLA may have poor capacity for detecting ploidy changes.

2′-Deoxycoformycin: negative (–S9/+S9: –/–). At dose levels up to 5 mg/ml, 2′-deoxycoformycin showed no mutagenic or cytotoxic responses in any experiment. This chemical is clearly positive in the BRM, CA and *in vivo* MN assay (Otsuka *et al*., 1991). Our negative results in the MLA may have been due to the exposure conditions.

1,3-Dimethylxanthine (theophylline): negative (–S9/+S9: –/–). 1,3-Dimethylxanthine was examined by one laboratory. It showed neither enough cytotoxicity nor increased mutation frequencies at doses up to 5 mg/ml with and without S9 mix.

Ethenzamide: positive (–S9/+S9: I/+). Because of ethenzamide's insolubility, all experiments were carried out in the presence of precipitate. In the presence and absence of S9 mix, cytotoxic and mutagenic responses were relatively dose-dependent within the acceptable dose ranges in lab B, while lab A did not observe any positive responses. Although the mutagenic response with S9 mix in lab B was obvious, we excluded these data because of an insufficient number of treatment doses (UE).

Methacrylic acid 2-hydroxypropyl ester: positive (–S9/+S9: +/+). Cytotoxicity and mutagenicity of methacrylic acid 2-hydroxypropyl ester were slightly greater in the presence of S9 mix. Statistically significant and reproducible responses were obtained in all experiments. This chemical induces structural and numerical changes in the CA but is negative in the BRM (Tanaka *et al*., personal communication).

α-Naphthoquinoline: positive (–S9/+S9: I/+). In the absence of S9 mix, α-naphthoquinoline induced a positive response in lab A but not in lab B, although it was tested in the same dose ranges. It is difficult to find effective doses for this chemical because no cytotoxic response was observed up to ~70 μg/ml and then severe cytotoxicity appeared in a narrow dose range. The positive result observed in lab A may have been due to a fortuitous choice of critical doses (90–120 μg/ml). In the presence of S9 mix, statistically significant responses were found in both laboratories, although different response degrees which may be attributable to the higher spontaneous mutation frequencies in lab A were observed.

Noscapine (1-narcotine): inconclusive (–S9/+S9: I/–). The chemical began to precipitate at 100 μg/ml in the absence S9 mix and 200 μg/ml in the presence of S9 mix. Lab A tested this chemical with and without S9 mix up to the doses showing remarkable RTG reduction. Lab B, on the other hand, tested the chemical at up to 5 mg/ml and it did not yield marked cytotoxicity in either RS or RTG. The different cytotoxic responses may be due to the fact that the two laboratories used different solvents. Positive mutagenic responses were obtained without S9 mix in lab A, but not in lab B. With S9 mix, no mutagenic responses were seen in either laboratory. Noscapine induces more numerical than structural changes (Gatehouse *et al*., 1991).

Oxytetracycline-HCl: inconclusive (–S9/+S9: I/I). In lab A, oxytetracycline-HCl was not mutagenic with or without S9 mix. In lab B, on the other hand, the chemical showed statistically significant positive responses in the presence and absence of S9 mix. Myhr *et al*. (1990) and McGregor,D.B. *et al*. (1991) reported that oxytetracycline-HCl was highly mutagenic to mouse lymphoma cells both in the presence and absence of S9 mix.

Phenacetine: inconclusive: (–S9/+S9: I/I). Phenacetine began to precipitate at ~1800 μg/ml both in the presence and absence of S9 mix. This precipitation might have resulted from different testing conditions between laboratories (lab A, up to 1800 μg/ml; lab B, up to 5000 μg/ml). The chemical was clearly positive with S9 mix but negative without S9 mix in lab A, while in lab B a positive response was found only in the absence of S9 mix. Because of the disparate results, this chemical was judged to be inconclusive. This chemical is negative in the standard BRM with rat S9 mix, but positive with hamster S9 mix (Weinstein *et al*., 1981). The result of the CA with Chinese hamster cells is clearly positive in the presence of rat S9 mix (Ishidate, 1987). Although the cytotoxic response was enhanced by rat S9 mix in this study, the enhancement of mutagenicity was not clear.

Phenylbutazone: positive (–S9/+S9: I/+). Phenylbutazone induced clear cytotoxic and mutagenic responses in lab B. The cytotoxicity increased steeply at 1200–1400 μg/ml without S9 mix and at 800–1000 μg/ml with S9 mix, which were mutagenic doses. Both laboratories tested this chemical in a narrow range of doses, but the results with and without S9 mix in lab A were not statistically significant. However, we considered the response with S9 mix in lab A to be dose-dependent and biologically significant (>3-fold increase).

Thiabendazole: negative (–S9/+S9: –/–). Thiabendazole, a spindle inhibitor, induced strong delayed cytotoxicity resulting in large differences between RS and RTG. Both laboratories tested this chemical up to doses showing marked cytotoxicity by RTG with and without S9 mix. No mutagenic responses were obtained in any experiments. Thiabendazole induces numerical changes in CHL cells in the CA but not structural changes (Ishidate, 1987). BRM data are unclear (Zeiger *et al*., 1988).

Triamterene: positive (–S9/+S9: +/–). In the absence of S9 mix, triamterene appeared to increase mutation frequencies dose-dependently up to 5–10 μg/ml and statistical significance was obtained in both laboratories, though a downturn phenomenon was observed at high doses. The compound was not mutagenic in the presence of S9 mix.

Trichloroethylene: positive (–S9/+S9: –/+). Because cytotoxic responses were stronger in the presence of S9 mix than in its absence, this chemical was metabolically activated by S9 mix. Negative responses were reproducibly observed without S9 mix in both laboratories. In the presence of S9 mix, both laboratories observed a statistically significant increase in mutation frequencies with S9 mix.

Vinblastine sulfate: inconclusive: (–S9/+S9: I/I). Vinblastine sulfate arrests cell division at metaphase by disrupting the mitotic spindle. This results in severe delayed cytotoxicity, which causes large differences between RTG and RS. Thus, both laboratories had difficulty finding effective doses. In the dose-finding tests, this chemical showed 10–20% RS at 40–100 μg/ml with and without S9 mix in both laboratories (data not shown). This dose range, however, turned out to be too toxic in the main experiments; there was no growth of cells during the expression time because of delayed cytotoxicity. Vinblastine sulfate was finally tested at <0.1 μg/ml. Thus, in the case of chemicals showing strong delayed cytotoxicity, RS is not useful as an indicator of cytotoxicity in the MLA.

Positive mutagenic responses were obtained with and without S9 mix in lab A but not in lab B. The chemical elicited mutagenic responses over a narrow dose range and it may be difficult to reproduce the results.

Zearalenone: negative: (–S9/+S9: –/–). Zearalenone was not mutagenic in any experiment. The lab A experiment with S9 mix appeared to be positive, but we excluded it from evaluation because of an insufficient number of treatment doses. McGregor *et al*. (1988b) tested zearalenone in the MLA with the agar method and concluded that it was negative, although one experiment out of five without S9 mix and one experiment out of three with S9 mix showed positive results. Zearalenone may have marginal mutagenic potential in the MLA.

The following three chemicals were judged to be inconclusive in the phase 1 study because of discrepant results between laboratories.

Bromodichloromethane: equivocal: (–S9/+S9: –/E). The cytotoxic and mutagenic responses were similar to those in the phase 1 study and a statistically significant response was found only in lab A with S9 mix. We finally judged this chemical to be equivocal.

Isophorone: negative: (–S9/+S9: –/–). Significant increases in mutation frequency were observed in all experiments but only at severely cytotoxic doses (<10% RS or RTG). Excluding those data, no biologically significant responses were obtained in lab A, although the response with S9 mix was statistically significant. Lab B data were excluded because of an insufficient number of treatment doses. Isophorone yielded severe delayed cytotoxicity leading to big differences between RS and RTG. According to our present criteria, isophorone was designated a negative chemical.

Tetrachloroethane: negative: (–S9/+S9: –/–). In the phase 1 study, this chemical showed a positive response with S9 mix in one laboratory. This response, however, was not reproduced in the phase 2 study: although the response was statistically significant in lab A with S9 mix, we considered the response not to be biologically significant. No statistically significant increases in mutation frequencies were obtained without S9 mix in lab A and with S9 mix in lab B. Thus we finally concluded tetrachloroethane to be negative.

Total evaluation

Table VIII presents the result of each chemical tested in this MLA collaborative study together with published results for them in the CA. A total of 41.2% of CA-positive chemicals (14/34) could not be detected by the MLA. On the other hand, 9.1% of MLA-positive chemicals (2/22) were not detected by the CA. In the present study, the majority of genotoxic chemicals induced positive responses in both the MLA and CA, but the finding that the MLA failed to detect >40% of the CA-positive chemicals is a serious problem. Thus, we could not conclude that the MLA is as sensitive as the CA.

One of the reasons that 14/34 CA-positive chemicals did not induce positive responses in the MLA may be that treatment duration was inadequate. In the CA, protocols with long continuous treatment (12, 24 or 48 h) together with pulse treatment (3 or 6 h) are used, while the standard MLA is performed only with pulse treatment (3 or 4 h).

Table VIII. Comparison of published CA results and the MLA results in the present collaborative study

MLA	CA			
	Positive	No.	Negative	No.
Positive	*N*-Aminoethyl ethanolamine		Cinnamyl anthranilate	
	Arsenic trioxide		Trichloroethylene	
	Benzene			
	Cadmium sulphate			
	Chlorodibromomethane			
	Cytosine arabinoside (Ara C)			
	Diethylstilbestrol (DES)			
	Ethenzamide			
	Eugenol			
	5-Florouracil (5-FU)			
	Griseofulvin			
	Hexamethyl phosphoramide	20		2
	Hydroxyurea			
	Methacrylic acid 2-hydroxypropyl ester			
	Methotrexate (MTX)			
	Monocrotaline			
	α-Naphthoquinoline			
	Pentachloroethane			
	Phenylbutazone			
	Triamterene			
Equivocal	Bromodichloromethane	1		
Inconclusive	Chlorendic acid		Benzyl acetate	
	Noscapine (1-narcotine)	4	Bisphenol A	3
	Phenacetine		Oxytetracycline-HCl	
	Vinblastine sulfate			
Negative	*p*-t-Butylphenol		Urethane	
	Colchicine			
	2′-Deoxycoformycin			
	Dideoxycytidine (DDC)			
	1,3-Dimethylxanthine	9		1
	Isophorone			
	Tetrachloroethane			
	Thiabendazole			
	Zearalenone			
Total		34		6

Underlined chemicals induced mainly numerical changes in the CA.

2′-Deoxycoformycin, noscapine and thiabendazole were negative or inconclusive in this MLA study. They were positive in the CA, however, with continuous treatment but not with pulse treatment. Thus, continuous treatment may be necessary for these chemicals to induce positive responses. In the standard MLA protocol (pulse treatment), 2′-deoxycoformycin, DDC, 1,3-dimethylxanthine and urethane did not cause sufficient cytotoxicity even up to the limit concentration (5 mg/ml). They might do so with continuous treatment. Thus, in order to compare the CA and MLA appropriately, we should consider using comparable exposures.

Eight chemicals (*N*-aminoethyl ethanol amine, chlorodibromomethane, DES, pentachloroethane, noscapine, *p*-t-butylphenol, tetrachloroethane and thiabendazole) induce mainly numerical chromosome changes in the CA. Four of them (*N*-aminoethyl ethanol amine, chlorodibromomethane, DES and pentachloroethane) were positive in the present MLA study. Although the reason for this is not clear, it suggest that the MLA may detect polyploidy and/or aneuploidy inducers.

Limitation of the data

This collaborative study was carried out with the participation of a large number of laboratories (>50) with different experience of this assay and so all of the data did not fit acceptable conditions for result evaluation and some test results were excluded based on our criteria of acceptable conditions described in Materials and Methods. Some data from borderline acceptable conditions were included for evaluation, because overall data sets for these chemicals were able to be judged [e.g. PE_2 of solvent control for bromodichloromethane (phase 1), –S9, lab A; benzyl acetate, +S9, lab A]. In addition, PE_0 of the positive control (MMS) did not decrease sufficiently in several experiments [bromodichloromethane (phase 1), –S9, lab B; chlorodibromomethane, –S9, lab B; MMC, –S9, lab A, lab B and lab C], as was also true for RTG (e.g. cadmium sulfate, –S9, lab A; chlorodibromomethane, –S9, lab A). Cytotoxicity of some chemicals did not hit a window of 10–20% RS or RTG [e.g. bromodichloromethane (phase 1), –S9, lab A and lab B; chlorodibromomethane, –S9, lab A and lab B; pentachloroethane, +S9, lab A and lab B; benzyl acetate, +S9, lab B]. It is recommended that some test results be confirmed, especially those mentioned above, by additional experiments. The overall conclusions drawn from the present study, however, will be consistent even if updating of individual evaluations on some chemicals are made in the future.

Protocol issues

In the present collaborative study we faced some problems of protocol and some on evaluating the results. These problems were discussed at the MLA workshop at Portland, OR, in 1994 (Clive *et al.*, 1995), but they were not completely resolved, especially for the microwell method. The major

issues are cytotoxicity range, definition of top concentration, acceptance of data with extraordinary plating efficiencies, acceptable range of spontaneous mutation frequencies, necessity for duplicate culture and duration of test chemical treatment.

The last issue in particular is important for both the agar and the microwell methods (see above). The standard MLA protocol requires only pulse treatment and a treatment time extended over two cell cycles is generally not needed for mammalian cell gene mutation assays (Aaron *et al*., 1994). Our present study, however, implied that some chemicals may need long-term continuous treatment to exhibit their mutagenicity in the MLA. We have since applied 24 h continuous treatment in the MLA for chemicals negative or inconclusive in the present study and the majority of them showed positive responses (Sofuni *et al*., 1997a; Honma *et al*., 1999).

The same protocol issues were re-discussed in 1996 at the second MLA workshop in Victoria, BC, Canada. Based on the workshop consensus agreements, a revised MLA protocol for the microwell method has been proposed (Sofuni *et al*., 1997b). The issue of the specificity of the MLA with 24 h continuous treatment was targeted in the third phase collaborative study and non-genotoxic chemicals were examined by both the microwell and soft agar methods to compare specificity of the standard short treatment and the 24 h continuous treatment (Müller *et al*., in preparation). All raw data are available in the Mutagenesis database. A copy can be obtained upon request to the Editor.

Conclusion

In the present collaborative study, the MLA was not as sensitive as the CA; >40% of CA-positive chemicals were not detected by the MLA. Our results suggest, however, that improvement of the MLA protocol, specifically the duration of treatment, may help to make the MLA more effective for detection of clastogens and spindle poisons.

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