any supportive data for the carcinogenic potential of this chemical as revealed in the long-term and promotion bioassays.

MX induced statistically significant positive responses in both initiation and promotion cell transformation assays. These findings agree with positive outcomes using a C3H 10T1/2 cell transformation assay system (21) and also give supportive information to the carcinogenicity of MX (2). However, tumours were not observed after *in vivo* inoculation of a large number of transformed cells harvested from the initiation assay in which MX was used as the initiator and TPA as the promoter. This may be owing to the fact that the BALB/c 3T3 cells of the transformation assay were derived from a BALB/c mouse strain as used for the tumourigenicity assay, or may be related to the rather short *in vivo* expression period. We did not perform a similar experiment using immune-deficient nude mice. However, within 2–4 months Boone and Jacobs reported the induction of tumours in BALB/c mice by inoculation of transformed cells (22). Although we carried out the study for 2–3 weeks, the period might have been too short to develop nodules. Cells isolated from transformed foci in the initiation assay did not induce any nodules after inoculation to BALB/c mice, the strain of mouse from which the transformation assay cells were derived. Taken together, we could not adjudge the malignancy of transformed cells induced by MX when used as an initiator.

The possibility of an *in vivo* promoting effect of MX was revealed by the positive result in the promotion assay using BALB/c 3T3 cells. Moreover, this result was supported by the demonstration that MX inhibited GJIC, which is a characteristic of many tumour promoters evaluated using the metabolic cooperation assay (8). The major role of GJIC is considered to be the maintenance of homeostasis in multicellular organisms, and it is believed that second messenger transfer through GJIC is important for cell growth control (23,24). Tumour-promoting chemicals such as TPA and analogues, DDT and aldrin inhibit GJIC (25–27), and this *in vitro* test for tumour promoters is recommended as a useful tool for detecting non-genotoxic carcinogens (28). This activity of MX in the current GJIC assay is consistent with a recent report on GJIC inhibition in BALB/c 3T3 cells (29).

MX appears to have weak genotoxicity in mammalian systems *in vivo*, and it is probable that the tumour promoting activity of MX is important for explaining its carcinogenic activity. Although many regulatory bodies assess chemical safety based on the dogma that genotoxic carcinogens do not have any threshold, we propose that risk assessment of MX takes into account the chemical's likely threshold as a tumour promoter.

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Received on January 5, 2005; revised on June 8, 2005; accepted on July 15, 2005

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Mutation Research 583 (2005) 133–145

Genetic Toxicology and Environmental Mutagenesis

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Evaluation of liver and peripheral blood micronucleus assays with 9 chemicals using young rats A study by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)—Mammalian Mutagenicity Study Group (MMS)

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Received 25 August 2004; received in revised form 18 February 2005; accepted 15 March 2005

Abstract

We conducted simultaneous liver and peripheral blood micronucleus assays in young rats with seven rodent hepatocarcinogens—4,4′-methylenedianiline (MDA), quinoline, *o*-toluidine, 4-chloro-*o*-phenylenediamine (CPDA), dimethylnitrosamine (DMN), *p*-dimethylaminoazobenzene (DAB), and di(2-ethylhexyl)phthalate (DEHP)—and two mutagenic chemicals—kojic acid and methylmethanesulfonate (MMS).

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1383-5718/$ – see front matter
doi:10.1016/j.mrgentox.2005.03.012

Quinoline, DMN, and DAB were positive in the liver assay, while *o*-toluidine, kojic acid, DAB, and MMS were positive in the peripheral blood assay. *o*-Toluidine, kojic acid, and DAB are reportedly negative in mouse bone marrow micronucleus assays, indicating a species difference.
Our results revealed a correlation between micronucleus induction in hepatocytes and hepatocarcinogenicity. This technique can be useful for the detection of micronucleus-inducing chemicals that require metabolic activation, and it enables simultaneous comparison of the micronucleus-inducing potential of chemicals in the liver and peripheral blood in the same individual.

Keywords: Young rat; Liver micronucleus; Peripheral blood micronucleus; Hepatocarcinogen

1. Introduction

In vivo rodent bone marrow (BM) micronucleus assay results correlate highly with carcinogenicity in many organs, but the test is rather insensitive to indirect and liver carcinogens [1]. The micronucleus-inducing potential of such chemicals can be detected in in vivo liver micronucleus assays [2–4], which can be conducted by the partial hepatectomy (PH) method [2,5,6], co-treatment with mitogens [7,8] or an in vivo/in vitro assay system [9]. These all have serious disadvantages. In the PH method, P-450, styrenemonooxygenase, epoxide hydrolase, and glutathione-*S*-epoxide transferase activity is decreased [10], and the method is time-consuming because it involves surgery. In the co-treatment method with mitogens, the mitogens can interact with the test chemicals [11]. The in vivo/in vitro assay system requires much effort, time, and expense.

Searching for better approach, we evaluated liver micronucleus assay that uses 4-week-old rats [11]. We evaluated the assay using the hepatocarcinogen diethylnitrosamine (DEN) [12]. In 4-week-old rats, not only liver growth but also P450 activity are at their maximum and glucuronic acid, sulfate, glutathione, and glycine conjugation levels are the same as in mature animals [13], as are the levels of hexobarbital hydroxylation, *N*-demethylation of ethylmorphine, *O*-demethylation of *p*-nitroanisole and hydroxylation of aniline [14]. Since the usefulness of this method has not been clearly demonstrated, we organized a collaborative study to evaluate it with nine model chemicals. We conducted the peripheral blood micronucleus assay [15,16] simultaneously to evaluate another organ in the same animal. Our results demonstrated the relationship in young rats between the hepatocarcinogenicity and hepatocyte micronucleus-inducing potential of the test chemicals.

2. Materials and methods

2.1. Collaboration

Eleven research laboratories collaborated in this study (Table 1).

2.2. Animals

Male Fischer F344 or SD rats, 3 weeks of age, were purchased from Charles River Japan Inc., and used at 4 weeks of age. The animals were housed under a 12-h light–dark cycle and allowed free access to commercial pellets and tap water.

2.3. Chemicals

4,4′-Methylenedianiline (MDA, CAS No. 101-77-9), kojic acid (CAS No. 501-30-4), quinoline (CAS No. 91-22-5), *o*-toluidine (CAS No. 95-53-4), 4-chloro-*o*-phenylenediamine (CPDA, CAS No. 95-83-0), and dimethylnitrosamine (DMN, CAS No. 62-75-9) were purchased from Wako Pure Chemical Industries Ltd.; *p*-dimethylaminoazobenzene (DAB, CAS No. 60-11-7), di(2-ethylhexyl)phthalate (DEHP, CAS No. 117-81-7), and methylmethanesulfonate (MMS, CAS No. 66-27-3) from Aldrich. Diethylnitrosamine (DEN, CAS No. 55-18-5) was purchased from Wako Pure Chemical Industries Ltd. or Tokyo Kasei Co. Ltd., and cyclophosphamide (CP, CAS No. 50-18-0) was purchased from ICN Biochemicals or Aldrich.

MDA, *o*-toluidine, CPDA, and DAB were suspended in olive oil, quinoline and DEHP in corn oil. Kojic acid was suspended in 1% sodium carboxymethylcellulose. DMN was dissolved in distilled water, MMS in physiological saline. DEN and CP, the positive control substances, were dissolved in distilled water, and the same lot chemical was used in all laboratories.

Table 1
Study participants

	Laboratory	Investigators
1	Biosafety Research Center, Foods, Drugs and Pesticides	Jin Tanaka
2	Hokko Chemical Industry Co. Ltd.	Yasushi Shimada
3	Ina Research Inc.	Hiroshi Suzuki[a], Kana Komatsu Akiko Koeda, Tadashi Imamura
4	Kaken Pharmaceutical Co. Ltd.	Junichi Yoshida
5	Kao Corporation	Naohiro Ikeda
6	Kissei Pharmaceutical Co. Ltd.	Kazuo Kobayashi, Yukari Terashima, Kaori Yasue
7	Mitsubishi Chemical Safety Institute Ltd.	Yukiko Saito
8	National Institute of Health Sciences	Takayoshi Suzuki, Makoto Hayashi
9	Nisshin Kyorin Pharmaceutical Co. Ltd.	Shigeki Hatakeyama
10	Sankyo Co. Ltd.	Toshiyuki Hagiwara, Ayumi Okazaki
11	Toa Eiyo Ltd.	Koko Nagaoka

[a] Chief study organizer.

2.4. Doses

We used 1/2 and 1/4 of the LD_{50} value of each chemical as the high and low dose. When the LD_{50} values were unclear, we estimated them by small-scale experiments according to the method of Lorke [17]. Negative control animals received the respective vehicle. Positive control animals received DEN at 40 mg/kg (liver micronucleus assay) or CP at 10 mg/kg (peripheral blood micronucleus assay). Each group consisted of four or five animals. Dosing was conducted once intraperitoneally or orally. With the exception of MMS, each chemical was evaluated by two laboratories.

2.5. Liver micronucleus assay

Rats were anesthetized with ethylether 3, 4 or 5 days after a single administration of test chemical or 5 days after administration of the negative or positive control chemicals. Hepatocytes were isolated by the collagenase perfusion method, rinsed with 10% neutral formalin two or three times, centrifuged at $50 \times g$ for 1 min, suspended in 10% neutral formalin, and stored under refrigeration. For staining, 10–20 μL of the suspension was mixed with an equal volume of acridine orange (AO)–4′6-diamidino-2-phenylindole dihydrochloride (DAPI) [12]. Approximately 10–20 μL of stained suspension was dropped onto a clean glass slide and covered with a cover slip (24 mm × 40 mm).

Microscopic preparations were evaluated with the aid of a fluorescence microscope (×400 or greater) with UV excitation. The number of micronucleated hepatocytes (MNHEPs) among 2000 hepatocytes (two fields) was recorded for each animal. MNHEPs were defined as hepatocytes with round or distinct micronuclei that stained like the nucleus, with the ≤1/4 diameter of the nucleus [7,18]. The number of mitotic cells per 2000 hepatocytes was determined.

2.6. Peripheral blood micronucleus assay

A small amount of blood was collected from a tail vessel on Day 2 after treatment, at which time most chemicals induce the maximum response [19]. It was stained by either of the following methods: (1) 5–10 μL was dropped on to AO-coated slides, covered with cover glasses, and stored in a deep freezer until analysis [15], or (2) 10 μL suspension was mixed with about 30 μL of 10% neutral formalin and stored at room temperature, the samples were mixed with an equal volume of AO solution (500 μg/mL) in the ratio of 1:1 and smeared on a glass slide immediately before analysis. Specimens were evaluated with the aid of a fluorescent microscope (×600 or greater) with B excitation. The number of micronucleated reticulocytes (MNRETs) among 2000 reticulocytes (RETs) and the number RETs among 1000 erythrocytes were recorded for each animal.

2.7. Statistical analysis

We determined the statistical significance of the incidence of micronucleated hepatocytes or reticulocytes using Kastenbaum and Bowman's method [20] and that of reticulocytes with the Student t-test.

3. Results

3.1. Liver micronucleus assay

Table 2 shows the results of the liver micronucleus assay. Quinoline, DMN, and DAB were positive in both

Table 2
Results of the liver micronucleus assay

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
MDA				
Lab 1				
0	4	5	0.11 ± 0.09	0.38 ± 0.35
200	4	3	0.14 ± 0.11	0.23 ± 0.10
	4	4	0.19 ± 0.13	1.35 ± 0.94
	4	5	0.16 ± 0.09	0.73 ± 0.49
300	4	3	0.10 ± 0.04	0.19 ± 0.18
	3	4	0.08 ± 0.10	0.43 ± 0.40
	4	5	0.10 ± 0.09	0.50 ± 0.19
400	2	3	0.38 ± 0.04[a]	0.23 ± 0.11
	2	4	0.20 ± 0.14	0.28 ± 0.32
	3	5	0.18 ± 0.08	0.20 ± 0.13
DEN*	4	5	1.21 ± 0.08[a]	0.25 ± 0.12
Lab 2				
0	4	5	0.00 ± 0.00	0.06 ± 0.09
150	4	3	0.00 ± 0.00	0.18 ± 0.12
	4	4	0.05 ± 0.04	0.20 ± 0.12
	4	5	0.03 ± 0.03	0.00 ± 0.00
300	3	3	0.10 ± 0.09	0.13 ± 0.13
	3	4	0.02 ± 0.03	0.07 ± 0.03
	4	5	0.08 ± 0.06	0.08 ± 0.03
DEN*	4	5	0.44 ± 0.20[b]	0.18 ± 0.09
Kojic acid				
Lab 1				
0	4	5	0.06 ± 0.03	0.66 ± 0.26
1000	4	3	0.06 ± 0.06	0.98 ± 0.43
	4	4	0.06 ± 0.05	0.85 ± 0.25
	4	5	0.08 ± 0.05	1.56 ± 0.14
2000	4	3	0.04 ± 0.05	0.44 ± 0.13
	4	4	0.08 ± 0.05	0.49 ± 0.35
	4	5	0.09 ± 0.08	1.04 ± 0.29
DEN*	4	5	0.66 ± 0.13[a]	0.65 ± 0.31
Lab 2				
0	5	5	0.07 ± 0.06	0.61 ± 0.22
1000	5	3	0.04 ± 0.02	0.50 ± 0.23
	5	4	0.10 ± 0.06	0.63 ± 0.17
	5	5	0.11 ± 0.07	0.93 ± 0.06
2000	5	3	0.08 ± 0.08	0.57 ± 0.21
	5	4	0.07 ± 0.03	0.57 ± 0.08
	5	5	0.05 ± 0.04	0.60 ± 0.12
DEN*	5	5	1.04 ± 0.16[a]	0.95 ± 0.08
Quinoline				
Lab 1				
0	4	5	0.09 ± 0.06	0.48 ± 0.33
75	4	3	0.16 ± 0.08	0.18 ± 0.10
	4	4	0.39 ± 0.20[a]	0.15 ± 0.07
	4	5	0.20 ± 0.09[b]	0.25 ± 0.27

Table 2 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
150	4	3	0.58 ± 0.46[a]	0.34 ± 0.21
	4	4	0.33 ± 0.09[a]	0.39 ± 0.15
	4	5	0.35 ± 0.22[a]	0.18 ± 0.16
DEN*	4	5	0.21 ± 0.14[b]	0.48 ± 0.33
Lab 2				
0	5	5	0.03 ± 0.03	0.44 ± 0.10
75	5	3	0.36 ± 0.10[a]	0.35 ± 0.12
	5	4	0.22 ± 0.06[a]	0.33 ± 0.10
	5	5	0.12 ± 0.06	0.36 ± 0.10
150	5	3	1.22 ± 0.09[a]	0.33 ± 0.06
	5	4	0.93 ± 0.22[a]	0.42 ± 0.15
	5	5	0.61 ± 0.07[a]	0.23 ± 0.06
DEN*	5	5	0.84 ± 0.12[a]	0.76 ± 0.10
o-Toluidine				
Lab 1				
0	4	5	0.05 ± 0.06	0.41 ± 0.09
300	4	3	0.10 ± 0.07	0.16 ± 0.20
	4	4	0.10 ± 0.09	0.09 ± 0.05
	4	5	0.11 ± 0.10	0.40 ± 0.37
600	4	3	0.05 ± 0.06	0.21 ± 0.17
	4	4	0.06 ± 0.05	0.08 ± 0.06
	4	5	0.01 ± 0.03	0.06 ± 0.08
DEN*	4	5	0.85 ± 0.17[a]	0.30 ± 0.11
Lab 2				
0	4	5	0.04 ± 0.05	0.59 ± 0.30
300	4	3	0.04 ± 0.07	0.28 ± 0.06
	4	4	0.05 ± 0.07	0.45 ± 0.17
	4	5	0.08 ± 0.12	0.66 ± 0.36
600	4	3	0.04 ± 0.08	0.27 ± 0.18
	4	4	0.04 ± 0.05	0.31 ± 0.14
	4	5	0.01 ± 0.03	0.64 ± 0.26
DEN*	4	5	0.68 ± 0.15[a]	0.46 ± 0.09
CPDA				
Lab 1				
0	4	5	0.11 ± 0.02	0.34 ± 0.13
150	4	3	0.11 ± 0.13	0.10 ± 0.07
	4	4	0.20 ± 0.09	0.18 ± 0.09
	4	5	0.15 ± 0.16	0.46 ± 0.42
300	4	3	0.21 ± 0.09	0.01 ± 0.03
	4	4	0.21 ± 0.13	0.04 ± 0.03
	4	5	0.23 ± 0.13	0.09 ± 0.12
DEN*	4	5	0.88 ± 0.34[a]	0.25 ± 0.18
Lab 2				
0	4	5	0.09 ± 0.05	0.65 ± 0.09
150	4	3	0.05 ± 0.00	0.48 ± 0.09
	4	4	0.08 ± 0.09	0.36 ± 0.09
	4	5	0.06 ± 0.05	0.76 ± 0.27

Table 2 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
300	4	3	0.06 ± 0.08	0.25 ± 0.09
	4	4	0.04 ± 0.05	0.28 ± 0.12
	4	5	0.10 ± 0.07	0.31 ± 0.20
DEN*	4	5	0.68 ± 0.17[a]	0.34 ± 0.13
DMN				
Lab 1				
0	4	5	0.04 ± 0.03	0.66 ± 0.30
5	4	3	0.36 ± 0.36[a]	1.03 ± 0.12
	4	4	0.35 ± 0.25[a]	0.76 ± 0.44
	4	5	0.33 ± 0.24[a]	0.41 ± 0.27
10	4	3	0.31 ± 0.23[a]	0.45 ± 0.27
	4	4	0.51 ± 0.33[a]	0.34 ± 0.10
	4	5	0.36 ± 0.19[a]	0.86 ± 0.17
DEN*	4	5	1.04 ± 0.33[a]	0.41 ± 0.18
Lab 2				
0	4	5	0.05 ± 0.00	0.78 ± 0.43
5	4	3	0.15 ± 0.07	0.38 ± 0.23
	4	4	0.34 ± 0.24[a]	0.55 ± 0.20
	4	5	0.36 ± 0.26[a]	0.73 ± 0.27
10	4	3	0.26 ± 0.09[a]	0.55 ± 0.36
	4	4	0.51 ± 0.20[a]	0.61 ± 0.22
	4	5	0.46 ± 0.09[a]	0.70 ± 0.27
DEN*	4	5	0.86 ± 0.30[a]	0.64 ± 0.33
DAB				
Lab 1				
0	4	5	0.03 ± 0.03	0.10 ± 0.07
71	4	3	0.19 ± 0.08[a]	0.18 ± 0.15
	4	4	0.11 ± 0.08[b]	0.23 ± 0.13
	4	5	0.08 ± 0.10	0.44 ± 0.10
142	4	3	0.35 ± 0.08[a]	0.21 ± 0.10
	4	4	0.16 ± 0.03[a]	0.11 ± 0.03
	4	5	0.14 ± 0.03[b]	0.13 ± 0.12
DEN*	4	5	0.63 ± 0.27[a]	0.63 ± 0.18
Lab 2				
0	4	5	0.19 ± 0.11	0.19 ± 0.18
120	4	3	0.36 ± 0.10[b]	0.59 ± 0.17
	4	4	0.48 ± 0.09[a]	0.29 ± 0.12
	4	5	0.61 ± 0.02[a]	0.39 ± 0.12
240	4	3	0.25 ± 0.06	0.28 ± 0.21
	4	4	0.41 ± 0.07[b]	0.36 ± 0.16
	4	5	0.38 ± 0.13[b]	0.44 ± 0.19
DEN*	3	5	0.95 ± 0.33[a]	0.15 ± 0.05
DEHP				
Lab 1				
0	4	5	0.05 ± 0.04	0.95 ± 0.19
1000	4	3	0.05 ± 0.04	0.33 ± 0.46
	4	4	0.05 ± 0.04	0.20 ± 0.08
	4	5	0.06 ± 0.09	0.43 ± 0.10

Table 2 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	Sampling time (days)	MNHEP (%) mean ± S.D.	Mitotic cell (%) mean ± S.D.
2000	4	3	0.04 ± 0.05	0.28 ± 0.49
	4	4	0.05 ± 0.04	0.23 ± 0.10
	4	5	0.05 ± 0.06	0.63 ± 0.34
DEN*	4	5	1.66 ± 0.24[a]	1.13 ± 0.13
Lab 2				
0	4	5	0.08 ± 0.06	0.73 ± 0.36
1000	4	3	0.04 ± 0.05	0.43 ± 0.06
	4	4	0.09 ± 0.06	0.65 ± 0.47
	4	5	0.06 ± 0.05	0.26 ± 0.06
2000	4	3	0.09 ± 0.08	0.23 ± 0.16
	4	4	0.09 ± 0.09	0.15 ± 0.06
	4	5	0.09 ± 0.09	0.28 ± 0.17
DEN*	4	5	0.98 ± 0.52[a]	0.41 ± 0.16
MMS				
Lab 1				
0	4	5	0.05 ± 0.06	0.56 ± 0.34
40	4	3	0.08 ± 0.06	0.89 ± 0.30
	4	4	0.01 ± 0.03	0.66 ± 0.21
	4	5	0.04 ± 0.05	0.66 ± 0.40
80	4	3	0.11 ± 0.05	0.60 ± 0.24
	4	4	0.11 ± 0.08	0.58 ± 0.40
	4	5	0.08 ± 0.05	0.80 ± 0.41
DEN*	4	5	0.88 ± 0.12[a]	0.69 ± 0.42

MDA, 4,4′-methylenedianiline; DEN*, diethylnitrosamine (as a positive control, 40 mg/kg); CPDA, 4-chloro-*o*-phenylenediamine; DMN, dimethylnitrosamine; DAB, *p*-dimethylaminoazobenzene; DEHP, di (2-ethylhexyl) phthalate; MMS, methylmethanesulfonate.

[a] Significantly different from the solvent control (Kastenbaum and Bowman test; $P<0.01$).

[b] Significantly different from the solvent control (Kastenbaum and Bowman test; $P<0.05$).

laboratories. Deaths occurred at 400 mg/kg of MDA in sampling groups as follows: two animals on Day 3, two on Day 4, and one on Day 5. Thus, the positive response in samples harvested on Day 3 was based on only two animals. At 300 mg/kg of MDA, one animal died on Day 4. MDA was negative at 300 mg/kg in each sampling day. The other five chemicals were negative.

The appearance of mitotic cells was confirmed in all laboratories with all chemicals.

3.2. Peripheral blood micronucleus assay

Table 3 shows the results of the peripheral blood micronucleus assay. Kojic acid, *o*-toluidine, and DAB were positive in both laboratories, MMS in the one that tested it. Quinoline was positive in one of the two laboratories. CPDA was significantly cytotoxic, decreasing the % RET in both laboratories.

4. Discussion

We conducted the liver and peripheral blood micronucleus assays concurrently in young rats with nine mutagenic and/or carcinogenic chemicals. Table 4 compares the data generated in this collaboration with published bone marrow and hepatocarcinogenicity data.

The mean incidence of MNHEPs (%) for 70 rats in the solvent control groups was 0.07 ± 0.06%. This low incidence suggests the robustness of the assay.

Quinoline, DMN and DAB were positive in the liver micronucleus assay. The MNHEP (%) induced by 150 mg/kg quinoline tended to decrease with sampling time in both labs. This may have been due to inhibition of hepatocyte proliferation, as evidenced by the decrease in mitotic cells. The same may be applicable to DAB at 142 mg/kg. Although a statistically significant increase in MNHEP (%) was induced by 400 mg/kg

Table 3
Results of the peripheral blood micronucleus assay

Chemical and dose (mg/kg)	No. of animals	MNRET (%) mean ± S.D.	RET (%) mean ± S.D.
MDA			
Lab 1			
0	4	0.06 ± 0.06	14.0 ± 1.3
200	4	0.09 ± 0.03	11.6 ± 1.2[d]
400	3	0.15 ± 0.05	14.4 ± 3.3
DEN*	4	0.05 ± 0.04	13.0 ± 1.7
CP**	4	0.73 ± 0.10[a]	9.0 ± 1.0[c]
Lab 2			
0	4	0.04 ± 0.03	
150	4	0.13 ± 0.03	
300	4	0.09 ± 0.08	NT
DEN*	4	0.01 ± 0.03	
CP**	4	0.93 ± 0.42[b]	
Kojic acid			
Lab 1			
0	4	0.19 ± 0.12	8.3 ± 1.2
1000	4	0.15 ± 0.04	6.2 ± 0.4[d]
2000	4	0.70 ± 0.24[a]	7.1 ± 0.6
DEN*	4	0.16 ± 0.08	7.6 ± 1.6
CP**	4	1.43 ± 0.24[a]	6.6 ± 0.6[d]
Lab 2			
0	5	0.07 ± 0.05	11.6 ± 0.3
1000	5	0.16 ± 0.04[a]	11.2 ± 0.6
2000	5	0.38 ± 0.04[a]	11.7 ± 0.8
CP**	5	0.93 ± 0.12[a]	10.4 ± 0.9
Quinoline			
Lab 1			
0	4	0.13 ± 0.03	4.2 ± 0.6
75	4	0.11 ± 0.06	5.0 ± 0.5
150	4	0.10 ± 0.00	4.0 ± 0.9
DEN*	4	0.10 ± 0.07	4.7 ± 0.2
CP**	4	1.14 ± 0.43[a]	3.6 ± 0.5
Lab 2			
0	5	0.07 ± 0.03	12.2 ± 0.4
75	5	0.08 ± 0.00	11.9 ± 0.3
150	5	0.17 ± 0.03[a]	11.3 ± 0.9
CP**	5	0.95 ± 0.06[a]	10.3 ± 1.0
o-Toluidine			
Lab 1			
0	4	0.08 ± 0.06	13.6 ± 0.7
300	4	0.25 ± 0.11[b]	17.1 ± 2.1[d]
600	4	0.36 ± 0.09[a]	18.7 ± 3.6
DEN*	4	0.08 ± 0.06	16.8 ± 3.5
CP**	4	0.86 ± 0.25[a]	13.7 ± 2.2
Lab 2			
0	4	0.21 ± 0.08	11.0 ± 1.5
300	4	0.19 ± 0.03	13.5 ± 2.7

Table 3 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	MNRET (%) mean ± S.D.	RET (%) mean ± S.D.
600	4	0.46 ± 0.11[a]	13.6 ± 3.7
CP**	4	0.93 ± 0.21[a]	9.2 ± 0.7
CPDA			
Lab 1			
0	4	0.05 ± 0.06	7.5 ± 2.4
150	4	0.10 ± 0.07	5.8 ± 1.4
300	4	0.08 ± 0.06	3.6 ± 0.4[d]
DEN*	4	0.05 ± 0.04	6.8 ± 1.0
CP**	4	0.90 ± 0.35[a]	6.4 ± 0.8
Lab 2			
0	4	0.05 ± 0.06	12.0 ± 2.2
150	4	0.08 ± 0.03	12.1 ± 3.1
300	4	0.13 ± 0.06	7.8 ± 0.5[d]
DEN*	4	0.03 ± 0.03	12.5 ± 1.3
CP**	4	0.76 ± 0.14[a]	12.3 ± 3.2
DMN			
Lab 1			
0	4	0.08 ± 0.06	17.7 ± 1.9
5	4	0.04 ± 0.05	17.4 ± 3.1
10	4	0.15 ± 0.08	16.9 ± 2.5
DEN*	4	0.03 ± 0.03	13.5 ± 2.3
CP**	4	1.01 ± 0.49[a]	12.6 ± 1.2[c]
Lab 2			
0	4	0.11 ± 0.09	16.1 ± 3.7
5	4	0.19 ± 0.14	15.6 ± 2.1
10	4	0.18 ± 0.09	15.4 ± 2.5
DEN*	4	0.25 ± 0.07	17.4 ± 1.7
CP**	4	0.89 ± 0.19[a]	15.4 ± 3.2
DAB			
Lab 1			
0	4	0.03 ± 0.05	
71	4	0.05 ± 0.06	NT
142	4	0.43 ± 0.23[a]	
CP**	4	0.64 ± 0.13[a]	
Lab 2			
0	4	0.05 ± 0.04	13.6 ± 2.1
120	4	0.03 ± 0.03	14.0 ± 2.8
240	4	0.25 ± 0.13[a]	21.9 ± 6.4[d]
CP**	4	0.55 ± 0.16[a]	11.8 ± 1.4
DEHP			
Lab 1			
0	4	0.14 ± 0.09	29.0 ± 2.5
1000	4	0.18 ± 0.16	21.7 ± 1.3[c]
2000	4	0.18 ± 0.06	22.3 ± 1.1[c]
DEN*	4	0.06 ± 0.05	14.7 ± 2.2[c]
CP**	4	1.23 ± 0.34[a]	13.1 ± 2.6[c]

Table 3 (*Continued*)

Chemical and dose (mg/kg)	No. of animals	MNRET (%) mean ± S.D.	RET (%) mean ± S.D.
Lab 2			
0	4	0.16 ± 0.05	23.1 ± 2.4
1000	4	0.25 ± 0.14	27.1 ± 7.4
2000	4	0.16 ± 0.05	23.1 ± 3.8
DEN*	4	0.19 ± 0.08	21.9 ± 3.3
CP**	4	1.06 ± 0.13[a]	21.9 ± 2.2
MMS			
Lab 1			
0	4	0.14 ± 0.10	8.5 ± 1.0
40	4	2.04 ± 0.79[a]	12.5 ± 4.8
80	4	0.96 ± 0.57[a]	3.8 ± 0.2[c]
DEN*	4	0.18 ± 0.05	12.4 ± 1.5[c]
CP**	4	1.54 ± 1.03[a]	7.8 ± 1.8

MDA, 4,4′-methylenedianiline; DEN*, diethylnitrosamine (the first positive control, 40 mg/kg); CP**, cyclophosphamide (the second positive control, 10 mg/kg); CPDA, 4-chloro-*o*-phenylenediamine; DMN, dimethylnitrosamine; DAB, *p*-dimethylaminoazobenzene; DEHP, di (2-ethylhexyl) phthalate; MMS, methylmethanesulfonate. NT: not tested.

[a] Significantly different from the solvent control (Kastenbaum and Bowman test; $P < 0.01$).

[b] Significantly different from the solvent control (Kastenbaum and Bowman test; $P < 0.05$).

[c] Significantly different from the solvent control (Student *t*-test; $P < 0.01$).

[d] Significantly different from the solvent control (Student *t*-test; $P < 0.05$).

MDA in samples harvested 3 days after dosing, the data were from only two animals. In conjunction with Lab 2 results, MDA was considered to be negative in this assay. Quinoline, DMN and DAB, are chemicals were also positive in the presence of metabolic activation in in vitro genotoxicity assays [21–23]. Quinoline and DMN induce hepatocellular carcinoma in mice and rats [24–26], while DAB induces hepatocellular carcinoma in rats, but not in mice [27]. Four chemicals that were negative in this study have been reported to be carcinogenic. MDA and CPDA induce hepatocellular carcinoma in male and female mice [28–30] and neoplastic nodules in rats [29–31]. *o*-Toluidine induces hepatocellular carcinomas and hemangiosarcomas in mice and multiple organs tumors in rats [32]. DEHP induces hepatocellular carcinoma in mice and rats [33], but this chemical, unlike quinoline, DMN, and DAB, is a peroxisome proliferator, not a genotoxic carcinogen [34]. So the negative results in this assay are understandable. MMS induces carcinomas in the nasal cavity, central nervous system, and injection sites [35], but did not induce micronuclei in this study. *O*-Alkylation

Table 4
Micronucleus assay results for nine chemicals in this study compared with results from published bone marrow and hepatocarcinogenicity assays

Chemical	MN		BM		Hepatocarcinogenicity	
	L	PB	Mouse	Rat	Mouse	Rat
MDA	–	–	– [1]	ND	+ [28,29]	+ [28,29]
Kojic acid	–	+	– [41,50]	ND	– [42]	ND
Quinoline	+	–, +	+ (–) [44,45]	ND	+ [25]	+ [24]
o-Toluidine	–	+	– [1]	ND	+ [32]	– [32]
CPDA	–	–	+ [16]	+ (–) [16]	+ [30,31]	+ [30,31]
DMN	+	–	– (+) [46,47]	ND	+ [26]	+ [26]
MMS	–	+	+ [16]	+ [16]	ND	– [35]
DAB	+	+	– [1]	ND	ND	+ [27]
DEHP	–	–	– [1]	ND	+ [33]	+ [33]

MN, micronucleus assay; L, liver; PB, peripheral blood; BM, bone marrow micronucleus assay; parentheses show peripheral blood micronucleus assay. ND, no data found.

is more efficient than *N*-alkylation in the formation of micronuclei [36–38], and considering that MMS causes primarily *N*-7-methylguanine formation [39], the negative results were expected. Regarding P450 levels in young rats, 1A2, 2A1, 2B1, 2B2, 2E1, 3A1, and 3A2 levels increase with age, and reaching a maximum at approximately 30 days. Thereafter, the levels are suppressed by growth hormones, and 2C7, 2C11, 2C12, and 2C22 levels increase [40]. Therefore, P450 changes may have affected the results of the assay. Quinoline, DMN, and DAB are clearly genotoxic in in vitro only following metabolic activation [21–23], which makes them suitable for this assay.

In summary, quinoline, DMN, and DAB, which are rat hepatocarcinogens, induced liver micronuclei in this study. MDA, kojic acid [41,42], *o*-toluidine, and CPDA, which possess weak or uncertain hepatocarcinogenic potential in rats, did not, nor did DEHP, a non-genotoxic rat hepatocarcinogen. All chemicals, except MMS, were evaluated in two laboratories with similar results, as noted.

Although we did not statistically analyze the incidence of mitosis, we observed an increase or decrease for each chemical. The mitotic index reflected only three time points and did not give any information about the total number of mitoses. Thus, a correlation between MNHEP (%) and mitotic index was not always evident. These results may reflect increased mitotic activity or cytotoxic action of test chemicals on dividing hepatocytes [43].

The mean incidence of MNRETs (%) for 70 rats in the solvent control groups was $0.10 \pm 0.08\%$. This low incidence suggests the robustness of the assay like the liver assay.

Quinoline at 150 mg/kg was positive in the peripheral blood micronucleus assay only in Lab 2. Inconsistent results for quinoline have been reported before: the compound was positive in mouse bone marrow micronucleus assays [44] and negative in transgenic mouse peripheral blood micronucleus assays [45]. Thus, quinoline induces micronuclei in the liver but may not in hepatopoietic tissue. DMN was negative in peripheral blood micronucleus assay in rats, but has been reported to be positive in transgenic mice [46]. It is also negative in the mouse bone marrow micronucleus assay [47]. These results may reflect the fact that *N*-nitroso chemicals are difficult to evaluate in bone marrow micronucleus assays [1]. The rate of *N*-hydroxylation of DAB is higher in rats than in mice [48]. *N*-hydroxylation of amino azo dyes generates mutagenic metabolites [49], which may yield different results. Although kojic acid and *o*-toluidine were positive in this study, they are negative in mouse bone marrow micronucleus assays [50,51]. The MNRET (%) for CPDA in Lab 2 tended to increase in a dose-dependent manner, though it did not reach a statistically significant level. Because of the MNRET (%) were not dose-dependent in Lab 1, CPDA was considered to be negative. Although, CPDA was negative in this assay, it is positive in mouse bone marrow assays [16]. Thus, species differences are evident for kojic acid, *o*-toluidine and CPDA. The results of MMS, a direct alkylating agent, were consistent with those of mouse/rat bone marrow micronucleus assays [16].

In the present study, we evaluated known hepatocarcinogenic chemicals for micronucleus-inducing effects in 4-week-old rats. For some chemicals, our peripheral blood results differed from those reported by others, perhaps because younger rats are more sensitive to mutagens [52]. Accordingly, the simultaneous liver and peripheral blood assay system may bring out different result to previously reported one. In this assay, rodent hepatocarcinogens have been mainly used. Further evaluation using other organ carcinogens should be performed to assess this system in future.

As shown with quinoline, DMN, and DAB, the liver MN assay detected chemicals that required metabolic activation. Thus, it could be used to confirm positive responses in in vitro genotoxicity assays. These assays could expand the information obtained, for example, in the in vivo/in vitro UDS (unscheduled DNA synthesis) assay or the in vivo single cell gel electrophoresis (Comet) assay.

In conclusion, this assay system enabled us to simultaneously detect hepatocyte and peripheral blood micronucleus induction in the same animal. We also obtained information on differences in clastogen sensitivity between rats and mice. More chemicals should be studied to elucidate the validity and the sensitivity of this assay system.

Acknowledgment

This article was communicated by the Mammalian Mutagenicity Study group (MMS) of the Environmental Mutagen Society of Japan (JEMS).

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Mutagenesis vol. 21 no. 1 pp. 11–13, 2006
Advance Access Publication 27 September 2005
doi:10.1093/mutage/gei053

Performance of flow cytometric analysis for the micronucleus assay—a reconstruction model using serial dilutions of malaria-infected cells with normal mouse peripheral blood

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To confirm the performance and statistical power of a flow cytometric method for scoring micronucleated erythrocytes, reconstruction experiments were performed. For these investigations, peripheral blood erythrocytes from untreated mice, with a micronucleated erythrocyte frequency of ~0.1% were combined with known quantities of *Plasmodium berghei* (malaria) infected mouse erythrocytes. These cells had an infected erythrocyte frequency of ~0.7%, and mimic the DNA content of micronuclei (MN). For an initial experiment, samples with a range of MN/malaria (Mal) content were constructed and analysed in triplicate by flow cytometry until 2000, 20 000 and 200 000 total erythrocytes were acquired. In a second experiment, each specimen was analysed in triplicate until 2000, 20 000, 200 000 and 1 000 000 erythrocytes were acquired. As expected, the sensitivity of the assay to detect small changes in rare erythrocyte sub-population frequencies was directly related to the number of cells analysed. For example, when 2000 cells were scored, increases in MN/Mal frequencies of 3.9- or 2.7-fold were detected as statistically significant. When 200 000 cells were analysed, a 1.2-fold increase was detected. These data have implications for the experimental design and interpretation of micronucleus assays that are based on automated scoring procedures, since previously unattainable numbers of cells can now be readily scored.

Introduction

From a statistical point of view, in order to achieve a higher power of detection, sample size should be increased. For many experimental situations, it is not always feasible to increase the number of subjects studied. When the event under consideration is rare as to cause appreciable scoring error, then an alternative would be to enhance the precision of each measurement. For example, in the rodent erythrocyte micronucleus assay, the evaluation of 2000 immature erythrocytes per animal and 5 animals per dose group represents commonly cited minimum values. Owing to the rarity of micronucleated cells, even this minimal assay design results in tedious and time-consuming efforts. The use of flow cytometry (1–3) realizes the ability to evaluate high numbers of erythrocytes, something that is impossible to achieve by manual microscopy. By reducing scoring error in this manner, flow cytometry has the potential to increase statistical power.

In the present study, we evaluated the relationship between statistical power to detect a rare erythrocyte sub-population, i.e. micronucleated or malaria-infected erythrocytes (MN/Mal), and the total number of erythrocytes analysed. These experiments were accomplished using a reconstruction model whereby known quantities of malaria-infected erythrocytes were added to blood from an untreated mouse. Malaria is a known model for micronucleated erythrocytes, as they endow the target cells of interest with a micronucleus-like DNA content (4,5). The samples were analysed by flow cytometry to measure the MN/Mal frequency through the interrogation of 2000 (2k), 20 000 (20k), 200 000 (200k) and 1 000 000 (1m) erythrocytes. The results presented here show the capability of flow cytometric technology to reduce scoring error, and also the extent to which this affects the ability to detect small changes to baseline micronucleus frequencies.

Materials and methods

Staining of blood specimens

Methanol-fixed blood from untreated and malaria-infected mice used in this study were two 'biological standards' which accompany the Mouse MicroFlow®PLUS kits (Litron Laboratories, NY). MicroFlow PLUS kits were the source of these specimens.

Before analysis, malaria-infected specimens and untreated mouse specimens were washed out of fixative with ~12 ml Hank's Balanced Salt Solution. Procedures for the 3-colour labelling technique which appear in the MicroFlow®PLUS instruction manual (version 031230) were scaled up ~7-fold in order to provide at least 10 ml each of control and malaria blood in a cell density range that is recommended for this assay (between ~2000 and 6000 events/s). Anti-CD71-FITC, anti-CD61-PE and all other flow cytometry reagents were also supplied in the kits. After the labelling procedures were accomplished, the cell density of the malaria-infected sample was adjusted so that it was equal to that of the control blood sample. Initial cell densities were measured with a Coulter Counter, model ZM. After adjustment with additional propidium iodide staining solution, equal cell densities were confirmed by Coulter Counter measurements. Normalization of cell densities was an important experimental design consideration, as this allowed us to calculate the expected MN/Mal frequencies in the diluted samples once the frequencies of the original control (0.10 and 0.09% for Experiments 1 and 2, respectively) and malaria-infected (0.67 and 0.70% for Experiments 1 and 2, respectively) samples were determined with high precision (i.e. control and malaria-infected %MN/Mal frequencies are the mean value of triplicate analyses with 1m erythrocytes per analysis).

Dilution of malaria blood specimen

Malaria-infected blood (Sample H) was diluted with control blood (Sample A) in the following ratios (v/v): 1:1 (Sample G), 1:3 (Sample F), 1:7 (Sample E), 1:15 (Sample D), 1:31 (Sample C) and 1:63 (Sample B). These blood specimens were stored at 4°C until flow cytometric analysis, which occurred on the same day. Each sample was analysed three times to evaluate reproducibility.

Flow cytometric analysis

All samples were analysed according to the MicroFlow® PLUS 3-colour technique. One deviation to the kit-supplied data acquisition and analysis template was that the frequency of erythrocytes with malaria or micronuclei was determined without restriction to CD71-expression level. That is, the

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Mal and MN frequencies measured and reported here are based on total peripheral blood erythrocytes. A second deviation from standard practices is that the default stop mode of 20 000 reticulocytes was not utilized. Rather, each specimen was analysed until the following number of erythrocytes were acquired: 2k, 20k and 200k erythrocytes in the first experiment and 2k, 20k, 200k and 1m erythrocytes in the second experiment.

Statistical analysis

The average of triplicate MN/Mal measurements associated with the control blood sample were compared with those associated with each of the other seven specimens by the Fisher's exact method. A P-value of 0.05 divided by 7 (number of sample groups) was considered evidence of a statistically significant difference. Expected versus observed MN/Mal frequencies were graphed for each measurement performed in the second experiment. Microsoft Excel (Microsoft Corp., Seattle, Washington) was used to determine a best-fit line. The associated equations and r^2 values were determined.

Results

Data from Experiments 1 and 2 are summarized in Table I and include the expected and observed MN/Mal frequencies. The MN/Mal frequencies shown are the average of triplicate analyses. As shown in Table I, for measurements based on 2k erythrocytes, samples with expected MN/Mal frequencies of 0.39 and 0.24% were found to be significantly different from control samples, in Experiments 1 and 2, respectively. These values correspond to fold increases of 3.9 and 2.7 for the first and second experiment, respectively. As more erythrocytes were analysed per sample, the detection limit was improved. For instance, measurements based on the evaluation of 200k erythrocytes per analysis show statistical significance for expected MN/Mal samples of 0.12 and 0.11%. These values correspond to an increase of ~1.2-fold. In fact for the second experiment, when a stop mode of 1m erythrocytes was investigated, statistical significance was observed between the control blood sample (0.09% MN/Mal) and the specimen with the lowest frequency of malaria (0.10% MN/Mal; $P = 0.00005$).

As an aid for visualizing the performance characteristics associated with the various number of cells analysed, scattergrams showing %MN/Mal measurement are presented (Fig. 1). Best-fit lines and equations are included with these graphs, and illustrate the degree to which the experimentally derived data agree with the linear relationship that is known to exist among MN/Mal frequencies for these specimens.

Discussion

To evaluate the performance and statistical power of a flow cytometric approach to score micronucleated erythrocytes, we performed a reconstruction model experiment by the serial dilution of malaria-infected mouse blood with normal mouse

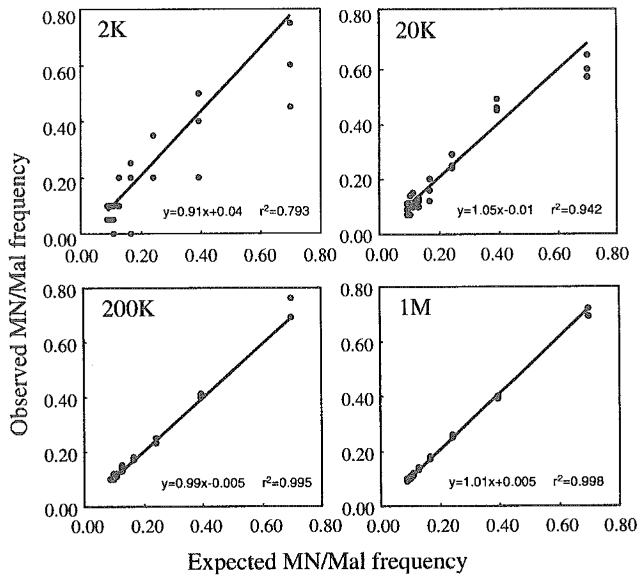

Fig. 1. Scattergram of expected versus observed MN/Mal frequencies. Each of three replicate measurements is plotted for these specimens. Best-fit linear lines are graphed, with associated equations. r^2 values document the degree of reproducibility observed.

Table I. MN/Mal frequencies (%) and P-values for comparisons with sample A

Sample	Expected (%)	Number of cells analysed/sample							
		2k		20k		200k		1m	
		(%)	P-value	(%)	P-value	(%)	P-value	(%)	P-value
Experiment 1									
A	0.10	0.07		0.09		0.10			
B	0.11	0.08	*0.50000*	0.11	*0.20764*	0.11	*0.13403*		
C	0.12	0.05	*0.77349*	0.10	*0.46272*	0.12	*0.00007*		
D	0.14	0.08	*0.50000*	0.13	*0.03463*	0.14	*0.00000*		
E	0.18	0.22	*0.02452*	0.21	*0.00000*	0.18	*0.00000*		
F	0.25	0.18	*0.05924*	0.30	*0.00000*	0.25	*0.00000*		
G	0.39	0.57	*0.00000*	0.36	*0.00000*	0.41	*0.00000*		
H	0.67	0.57	*0.00000*	0.73	*0.00000*	0.67	*0.00000*		
Experiment 2									
A	0.09	0.08		0.09		0.10		0.09	
B	0.10	0.08	*0.62305*	0.11	*0.20374*	0.11	*0.04822*	0.10	*0.00005*
C	0.11	0.05	*0.85547*	0.12	*0.06479*	0.12	*0.00301*	0.11	*0.00000*
D	0.13	0.13	*0.29053*	0.12	*0.08887*	0.14	*0.00000*	0.13	*0.00000*
E	0.17	0.15	*0.21198*	0.16	*0.00038*	0.17	*0.00000*	0.17	*0.00000*
F	0.24	0.30	*0.00531*	0.26	*0.00000*	0.24	*0.00000*	0.25	*0.00000*
G	0.40	0.37	*0.00076*	0.47	*0.00000*	0.41	*0.00000*	0.40	*0.00000*
H	0.70	0.60	*0.00000*	0.61	*0.00000*	0.71	*0.00000*	0.70	*0.00000*

Shading indicates those samples that are significantly different from respective control samples.

blood. As expected, the present results illustrate that the power of rare event detection is directly related to the number of cells analysed per specimen. By analysing 3m (triplicate of 1m) cells per group, 0.10% is significantly different ($P = 0.00005$) when compared with 0.09%. Even so, it must be appreciated that the biological significance of minute changes must be considered in addition to statistical significance.

Previously, we have shown that individual differences were negligible in the mouse micronucleus assay when 1000 cells per animal and 5 or 6 mice per dose group were analysed (6–8) and the statistical unit for the evaluation can be assigned to a cell but not to an animal. According to the present results and also results by Asano *et al.* (9), the variability of the data was high when 2k cells were analysed. Under these circumstances, the difference among animals is not apparent, as they are likely to be smaller than the scoring error. While, in the case of the present malaria dilution experiments, when 200k or 1m cells per sample were analysed, the scoring error decreased and converged to a value. This, however, is not true in the case of the actual micronucleus assays using model chemicals (9). When 200k or 1m immature erythrocytes were analysed, differences between individual animals became apparent and there was data variability within each dose group. Therefore, even if the experimental size in the animal experiments is increased, we cannot expect the same increment of detecting power. This finding suggests that optimizing the statistical procedure also includes evaluating individual differences.

Based on the present results, we confirm the accuracy and high performance of the micronucleus assay system using flow cytometry and we propose that the number of reticulocytes analysed for the micronucleus assay using flow cytometry be a minimum of 20k. We suggest that the analysis of 20k reticulocytes is approximately equivalent to the manual microscopic analysis according to test guideline OECD 474 (9,10). We anticipate that the experimental size of the MN assay will be recommended and set by expert committees based on the evaluated data. In addition to statistical sensitivity, biological variability between animals and as a consequence of treatment should also be considered. There appears to be diminishing value to analyses based on 200k or even 1m per animal. These may be useful in certain special circumstances, for instance when looking for evidence of threshold or practical threshold effects (9).

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Received on May 23, 2005; revised on July 21, 2005; accepted on July 22, 2005

Inhibitory effects of NADH/NADPH in S9 mix on photo-mutagenicity of thiabendazole following UVA-irradiation in *E. coli*

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Summary

Thiabendazole (TBZ), a post-harvest fungicide commonly used on imported citrus fruits, exhibited photo-mutagenicity following UVA-irradiation (320−400 nm) in Trp^+ reverse mutation assay using *Escherichia coli* WP2*uvrA*/pKM101 strain. The photo-mutagenicity was not observed in the presence of S9 mix, a rat liver homogenate microsome fraction with co-factors for metabolic activation. We found that NADH and NADPH used as co-factor in the S9 mix efficiently suppressed the photo-mutagenicity of TBZ. This evidence strongly suggested that non-mutagenicity in the presence of S9 mix was not due to the metabolic detoxification of TBZ or the scavenging of UVA-activated TBZ by macromolecules in the S9 mix. Rather quenching effect of NADH and NADPH (λmax=338 nm) may be more responsible for suppression of UVA-activation of TBZ, because oxidized forms of NAD^+ and $NADP^+$ did not show inhibitory effects. Mutagenicity of the UVA-irradiated photo-mutagens such as angelicin and chlorpromazine was also suppressed by the addition of NADH or NADPH. Our present results suggest the possible underestimation in risk evaluation for photo-mutagenic compounds when they are assayed in the presence of S9 mix.

Keywords: photo-mutagenicity, TBZ, chlorpromazine, UVA, S9 mix

Introduction

Benzimidazole fungicides such as thiabendazole (TBZ) and benomyl are widely used because of their non-toxicity to higher plants. TBZ is approved in Japan as a post-harvest fungicide for imported citrus fruits during transport and storage. Although TBZ is reported to be cytotoxic to the spindle apparatus and mitosis in mammalian cells (Styles and Garner, 1974; Mudry de Pargament et al., 1987; Parry and Sors, 1993), it is not mutagenic in bacterial reverse mutation tests with or without metabolic activation (Cancer Assessment Document, EPA, 2000). We recently reported that TBZ shows potent mutagenicity following UVA-irradiation (320−400 nm) for 10 min at 250 $\mu W/cm^2$ and that Trp^+ reverse mutations in *Escherichia coli* WP2*uvrA*/pKM101 strain is more sensitive than His^+ reverse mutations in *Salmonella typhimurium* strains TA100 and TA98 (Watanabe-Akanuma et al., 2003). The predominant mutations induced by UVA-activated TBZ were G:C→A:T transitions and A:T→T:A transversions. Since pre-irradiated TBZ solution just before adding bacterial cells did not show any mutagenicity, it seems likely that the photo-mutagenic TBZ products are unstable and/or rapidly react with other molecules before being incorporated into the cells (Watanabe-Akanuma et al., 2003). The photo-mutagenicity of TBZ in the *E. coli* strain was also observed with a fluorescent lamp, probably due to a low dose of UVA form the lamp (unpublished observation). For further investigation of the photo-mutagenic activation of TBZ by UVA-irradiation, we have conducted a screening assay to find effective inhibitors. As far as tested, several scavengers for reactive oxygen species such as ethanol, dimethyl sulfoxide, mannitol, histidine, ascorbic acid, epigallocatechin did not show apparent inhibitory effects (unpublished observation). On the other hand, the

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Received: October 8, 2004, revised: January 4, 2005,
accepted: January 6, 2005

photo-mutagenic activation of TBZ was not observed in the presence of exogenous metabolic activation system (S9 mix) in our preliminary study. We, therefore, tested the possible suppressing effect of macromolecules like DNA, proteins, and enzymes as well as S9 fraction (rat liver microsome fraction) in this study. We report here that photo-mutagenicity of TBZ was completely abolished by the addition of NADH and NADPH which were commonly used as co-factors to prepare the S9 mix, but not by the S9 fraction or other co-factor components, suggesting a mechanism other than metabolic detoxification of TBZ.

Materials and Methods

Bacterial strain, media, and chemicals

E. coli strain WP2*uvrA*/pKM101 (*trpE65*, *uvrA155*, *malB15*, *lon-11*, *sulA1*) was used for Trp^+ reverse mutation assay. Bacteria were cultured in Oxoid nutrient broth No. 2 at 37℃. Minimal glucose agar plates, and top agar for the Trp^+ reversion assay were described previously (Watanabe-Akanuma et al., 2003). S9 mix consisted of 10% rat liver homogenate S9 fraction, 4 mM NADH, 4 mM NADPH, 5 mM glucose-6-phosphate (G-6-P), 33 mM KCl, 8 mM $MgCl_2$, 100 mM sodium phosphate buffer (pH 7.4), according to the Guideline for Screening Toxicity Testing in Chemicals, Japan (1997).

Thiabendazole [TBZ, 2-(thiazol-4-yl)benzimidazole, CAS Registry number 148-79-8, (chemical structure in Fig. 1)], chlorpromazine hydrochloride [CAS No. 69-09-0, (chemical structure in Fig. 3 *right*)], dimethyl sulfoxide (DMSO), L-cysteine, pyridoxal hydrochloride, bovine serum albumin (BSA) and salmon sperm DNA were purchased from Wako Pure Chemical Industries, Japan. β-NADH and β-NADPH (reduced forms), β-NAD^+ and β-$NADH^+$(oxidized forms), G-6-P, S9 fraction, and co-factors mix solution were purchased from Oriental Yeast Co., Japan. Angelicin [CAS No. 523-50-2, (chemical structure in Fig. 3 *left*)], glutathione (reduced forms), and superoxide dismutase (SOD) from bovine erythrocytes were purchased from Sigma-Aldrich Co. MO, USA. Catalase from beef liver was purchased from Roche Diagnostics Co., IN, USA. UV absorption spectra from 280−400 nm of NADH, NADPH, and TBZ were measured using a U-2000A spectrophotometer (Hitachi Ltd., Japan).

UVA-irradiation

A black-light fluorescent lamp (National Black Light Blue, FL15BL-B, 15W, Matsushita Electric Industrial Co., Japan) that emitted wavelengths of 300−400 nm was used as the UVA source. To filter out UVB wavelengths below 320 nm, which are weakly mutagenic to the tester strain, a 5-mm thick soft glass plate was used. UVA was irradiated from a distance of 22 cm at 250 $\mu W/cm^2$(UVX Radiometer, Model UVX-36, Ultra-Violet Products, Upland, CA, USA) for 10 min on a 24-well multiplate with lid.

Mutagenicity assay

Bacteria were grown overnight in nutrient broth to a density of $1-3\times10^9$ cells/mL. A 0.1 mL aliquot of overnight culture was added to each well of a 24-well multiplate containing 0.5 mL of either S9 mix or 100 mM sodium phosphate buffer (pH 7.4). TBZ solution (2.5−20 μL) dissolved in DMSO at a concentration of 10 mg/mL was added at doses of 25−200 μg, and mixed well by pipetting. There was no precipitation of TBZ. The mixture was irradiated by UVA for 10 min at room temperature. Photomutagens, angelicin (Venturini et al., 1980; 1981) and chlorpromazine (Ciulla et al., 1986; Oppenländer, 1988; Gocke, 1996) dissolved in DMSO and sterile water respectively, were also used. In other experiments, one of the following compounds was added to the well containing phosphate buffer, TBZ, and bacteria: 1−100 μg of DNA, BSA, catalase, and SOD (10 μL of 0.1−10 mg/mL solutions), 1−50 μL of S9 fraction, 0.1−3 μmol NADH, NADPH, NAD^+, and $NADP^+$(10 μL of 10−300 μmol/mL solutions). After the mixtures were irradiated by UVA for 10 min, they were transferred to 2 mL of molten top agar in a test tube kept at 46℃, and immediately poured onto a minimal glucose agar plate. Plates were incubated for 2 days at 37℃ and the number of Trp^+ revertant colonies was counted. Experiments were conducted with duplicate plates for each dose and triplicate plates for the control. Data presented in the figures are the averages of duplicate or triplicate plates.

Results

Suppressing effect of macromolecules such as proteins and DNA in the irradiation mixture on photo-mutagenicity of TBZ was first investigated. At a dose of 150 μg (0.75 μmol) TBZ, UVA-irradiation for 10 min caused about 7-fold increase in the number of Trp^+ revertants of WP2*uvrA*/pKM101 over the corresponding control plates, while neither TBZ nor UVA alone was mutagenic (Table 1). Addition of sermon sperm DNA, BSA, reactive oxygen eliminating enzymes (SOD and catalase) up to a dose of 100 μg did not cause any suppressing effects. On the other hand, photo-mutagenicity of TBZ was largely reduced in the presence of S9 mix, but not S9 fraction alone (Table 1), suggesting either that the UVA-activated TBZ easily reacted with compounds in the S9 mix before entering the cells, or that the metabolites of TBZ were no longer photo-mutagenic. Further investigations, however, revealed that the photo-mutagenicity of TBZ was completely inhibited by the addition of 0.5 mL of co-factors solution alone as shown in Fig. 1. The results implied that the lack of photo-mutagenicity of TBZ in the presence of S9 mix was not due to simple metabolic detoxification of TBZ. Among the ingredients (G-6-P, NADH, NADPH, KCl, and $MgCl_2$) of co-factors, NADH and NADPH were found to be responsible for the suppressing effects. As