3.38, 4.50, 6.00 and 8.00 μg/ml. The culture medium was removed 72 h later, and the cells were fixed with 10% neutral buffered formalin and stained with 0.1% crystal violet for 30 min. For cytotoxicity testing with the promotion assay protocol, cells were seeded as above and MEM was replaced with DME/F12 medium 48 h after seeding. The cells were then treated with 1.68, 2.10, 2.62, 3.28, 4.10, 5.12 and 6.40 μg/ml of MX, and fixed and stained 96 h after cell seeding in the same manner as for the initiation assay cytotoxicity test. Extractant (1.5 ml) was placed in each well for 10 min and then absorption was measured with a spectrophotometer set at 580 nm. Cell survival at each dose was calculated relative to the negative vehicle control group. The MX concentration that inhibited cell growth by 50% (IC_{50} value) was calculated using the Probit method and approximately twice the IC_{50} was selected as the highest concentration for the cell transformation assays.

Cell transformation assays

In the initiation assay, 1.2×10^4 cells were seeded into 60 mm diameter culture dishes, 12 dishes per concentration, and control groups. After 24 h incubation, DMSO as negative control, MX as the initiator (1.64, 2.05, 2.56, 3.20 and 4.00 μg/ml) or MCA as a positive control (0.2 μg/ml) was added. Seventy-two hours after treatment, MEM was replaced with fresh DME/F12. On the 7th day after the beginning of treatment, TPA (0.1 μg/ml) or DMSO was added to cultures as the first promoter treatment. For the second and third promoter treatments, TPA or DMSO was added on the 11th and 14th day, respectively.

In the promotion assay, MCA (0.2 μg/ml, as the initiator) or DMSO was added 24 h after seeding 1.2×10^4 cells per dish. On the 4th day, MEM was replaced with fresh DME/F12. On the 7th day, saline, MX (0.156, 0.313, 0.625, 1.25 and 2.50 μg/ml) or TPA (positive control; 0.1 μg/ml) was added to the culture. MX and TPA were also added on the 11th and 14th day.

The cells were fixed with methanol and stained with 2.5% Giemsa solution on the 25th day for both assays. The foci that met the following criteria were counted as transformant: (i) 2 mm or more in diameter, (ii) criss-cross growth pattern, (iii) layering of cells and (iv) deep basophilic staining.

Tumourigenicity of transformed cells

Six-week-old male BALB/c CR mice were purchased from Japan Slc, Inc. (Shizuoka, Japan), and were quarantined and acclimated to the testing facility for 1 week. They were given pelleted diet (MF: Oriental Yeast Co., Ltd) and tap water *ad libitum* through the acclimation and assay periods.

At the end of the transformation assay with MX (4.0 μg/ml as initiator and 2.5 μg/ml as promoter; experimental data not shown), cultures were washed once with Dulbecco's PBS. Cells were isolated from transformed foci by trypsinization and mass cultured. An aliquot of 0.2 ml of cell suspension (1×10^6 cells for Experiment 1 and 1.5×10^6 cells for Experiment 2) was injected subcutaneously into the cervical region of the BALB/c CR mice. In both experiments, the cells isolated from the transformed foci in the negative control groups were inoculated into three animals and cells isolated from MX-induced transformed foci were inoculated into four animals. All animals were examined 2 weeks (Experiment 1) or 3 weeks (Experiment 2) after inoculation.

Metabolic cooperation assay

6-Thioguanine (6-TG) sensitive V79 cells (6-TG^s; 4×10^5 cells) and 6-TG resistant cells (6-TG^r; 200 cells) were co-cultured to evaluate the inhibition of metabolic cooperation (5 dishes per concentration). For calculating cytotoxicity 200 V79 [6-TG^s or 6-TG^r] cells alone were plated (3 dishes per concentration). Cells were treated with either MX or TPA 4 h after seeding. The 6-TG (10 μg/ml) was added 15 min after MX or TPA treatment and cells cultured for an additional 3 days before the medium was replaced with fresh medium containing only 6-TG; the cells were cultured another 4 days. The cells were fixed in ethanol and stained with 0.1% crystal violet for 10 min. Colonies with 50 or more cells were counted. These colonies developed from cells that were either not in GJIC contact with 6-TG^s cells or were in contact but then 'rescued' by GJIC inhibition from test chemical action. The assay is based on toxicity of 6-TG to 6-TG^sV79 cells (HGPRT^+), non-toxicity of 6-TG to 6-TG^r mutant V79 cells (HGPRT^-), with toxicity to these latter cells if in GJIC contact with HGPRT^+ cells, which transfer the HGPRT-catalysed toxic 6-TG metabolite via gap junctions to the HGPRT^- cells. Inhibition of GJIC rescues the contacting mutant cells to allow their clonal expansion (20).

Statistical analysis

The percentage of dishes with foci and the mean number of foci per dish were analysed using Fisher's exact test and the Wilcoxon's rank sum test, respectively.

In the metabolic cooperation assay, the number of 6-TG^r colonies was analysed for difference from the negative control group using Dunnett's test.

Results

Initiation assay

In the cytotoxicity assay for dose-range finding, a concentration-dependent decrease in cell survival was observed with MX treatment (Figure 1). An IC_{50} value using Probit's method was calculated to be 1.92 μg/ml. In the negative control group (saline initiation–TPA promotion) the mean number of transformed foci per dish was 0.50 and the percentage of dishes with foci was 41.7% (5 of 12 dishes, Table I). When MX was used as an initiator at 1.64, 2.05, 2.56, 3.20 and 4.00 μg/ml, and with DMSO post-treatment, no significant increase in transformation was observed. In the five groups treated with 1.64–4.00 μg/ml MX (as initiator) and TPA (0.1 μg/ml, as promoter) the numbers of foci per dish were
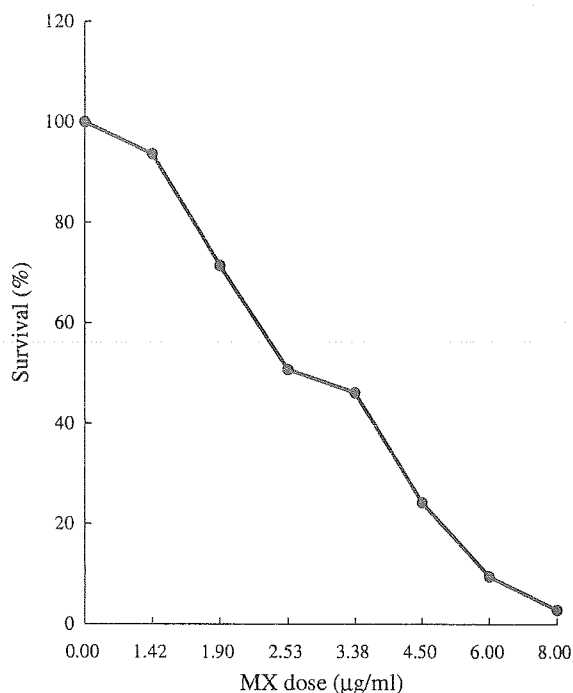

Fig. 1. MX dose–response for cell survival (Initiation protocol).

Table I. Initiating activity of MX in the two-stage transformation assay

Initiator	Conc. (μg/ml)	Survival (%)	Promoter	Conc. (μg/ml)	Mean foci/dish	Number of dishes with foci
Saline		100.0	DMSO		0.09	1/11 (9.1%)
MX	1.64	73.8	DMSO		0.08	1/12 (8.3%)
	2.05	59.2			0.25	2/12 (16.7%)
	2.56	33.7			0.00	0/12 (0.0%)
	3.20	20.5			0.33	3/12 (25.0%)
	4.00	18.8			0.25	3/12 (25.0%)
MCA	0.2	57.6	DMSO		0.58*	7/12* (58.3%)
Saline		–	TPA	0.1	0.50	5/12 (41.7%)
MX	1.64	–	TPA	0.1	0.58	6/12 (50.0%)
	2.05	–			1.25	8/12 (66.7%)
	2.56	–			1.92*	10/12 (83.3%)
	3.20	–			1.82*	9/11 (81.8%)
	4.00	–			3.64**	11/11** (100%)
MCA	0.2	–	TPA	0.1	7.58**	12/12** (100%)

$^*P \leq 0.05$, $^{**}P \leq 0.01$, significant difference from control (Wilcoxon's rank sum test for mean number of foci and Fisher's exact test for the percentage of dishes with foci).
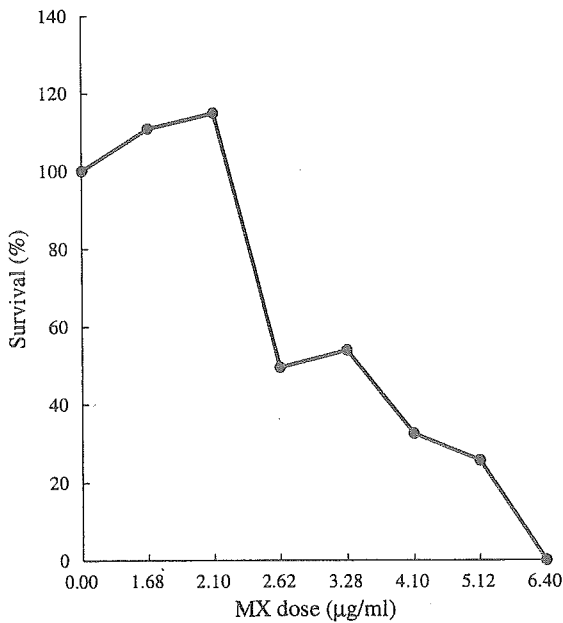Conc., concentration.

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Fig. 2. MX dose–response for cell survival (Promotion protocol).

Table II. Promoting activity of MX in the two-stage transformation assay

Initiator	Conc. (μg/ml)	Promoter	Conc. (μg/ml)	Survival (%)	Mean foci/dish	Number of dishes with foci
DMSO		Saline		100.0	0.08	1/12 (8.3%)
DMSO		MX	0.156	98.5	0.00	0/12 (0.0%)
			0.313	103.2	0.08	1/12 (8.3%)
			0.625	93.3	0.00	0/12 (0.0%)
			1.25	90.6	1.10	4/10 (40.0%)
			2.50	90.2	0.90**	7/10** (70.0%)
MCA	0.2	Saline		–	0.25	3/12 (25.0%)
DMSO		TPA	0.1	160.8	0.25	3/12 (25.0%)
MCA	0.2	MX	0.156	–	0.25	3/12 (25.0%)
			0.313	–	0.92	5/12 (41.7%)
			0.625	–	1.33*	10/12** (83.3%)
			1.25	–	3.40**	10/10** (100%)
			2.50	–	6.18**	11/11** (100%)
MCA	0.2	TPA	0.1	–	2.73**	10/11** (90.9%)

*$P \leq 0.05$, **$P \leq 0.01$, significant difference from control (Wilcoxon rank sum test for mean number of foci and Fisher's exact test for the percentage of dishes with foci).
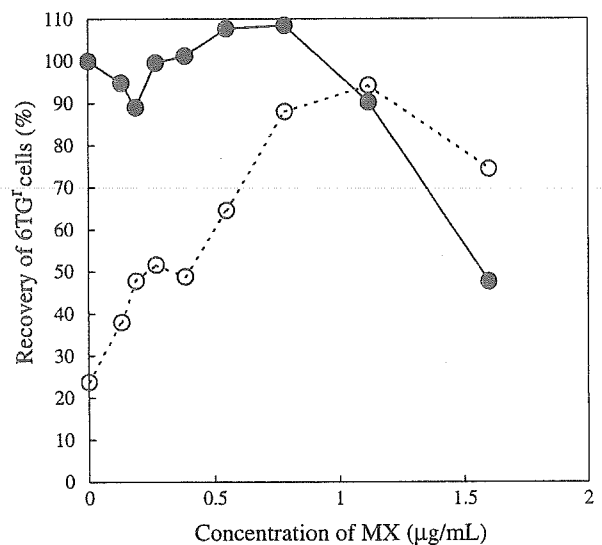Conc., concentration.

0.58, 1.25, 1.92, 1.82 and 3.64, respectively. A statistically significant increase ($P < 0.05$) was observed at MX ≥ 2.56 μg/ml compared with the negative control group. The number of dishes with foci also increased in a concentration-dependent manner and foci were observed in all dishes at the highest concentration (4.00 μg/ml) group. A large number of foci were induced in the positive control and MCA initiation-TPA promotion group; the mean number of foci per dish was 7.58, and all dishes contained foci.

Promotion assay

In the cytotoxicity assay, a concentration-dependent decrease in cell survival with MX treatment was observed (Figure 2). The IC_{50} value was 3.37 μg/ml. The experimental doses for promotion assay included at least 2 doses expecting to have $\geq 90\%$ cell survival rate. In the negative control group (MCA initiation-saline promotion), the mean number of foci per dish was 0.25 and the percentage of dishes with foci was 25% (3 out of 12 dishes, Table II). After initiation treatment with DMSO, cells were treated with MX at 0.156, 0.313, 0.625, 1.25 and 2.50 μg/ml as the promoter. The numbers of foci were 0.00, 0.08, 0.00, 1.10 and 0.90 per dish, respectively, with only the highest dose eliciting a significant increase in dishes with foci (Table II). In the groups treated with MCA (0.2 μg/ml) and MX at the above five concentrations, the numbers of foci were 0.25, 0.92, 1.33, 3.40 and 6.18 per dish, respectively. A statistically significant increase ($P < 0.05$) was observed at concentrations ≥ 0.625 μg/ml compared with the negative control group. The number of dishes with foci also increased concentration-dependently, and foci were observed in all dishes of the 1.25 and 2.50 μg/ml groups. The positive control group (MCA initiation-TPA promotion) confirmed the effectiveness of MCA/TPA in this cell transformation assay.

Tumourigenicity assay

Gross examination of the mice necropsied in tumourigenicity experiments 1 and 2 did not reveal any visible nodules or tissue masses in any organs of any animals.

Fig. 3. Inhibition of intercellular metabolic cooperation in V79 cells by MX. For calculation of cell survival, 200 6-TG^r cells were cultured (filled circle). Recovery of 6-TG^r cells (open circle) under conditions of metabolic cooperation (4×10^5 6-TG^s cells and 200 6-TG^r cells).

Inhibition of metabolic cooperation assay

The mean number of 6-TG^r colonies increased dose-dependently in the MX-treated groups (Figure 3), indicating an inhibition of GJIC by MX. This occurred at non-cytotoxic concentrations. The mean number of 6-TG^r colonies at 1.12 μg/ml of MX was 188.4 ($=94.2\%$) compared with 47.6 ($=23.8\%$) in the negative control. In the positive control (TPA-treated) group the mean number of 6-TG^r colonies was 159.4 ($=79.7\%$).

Discussion

From a public health viewpoint it is important to understand the toxicology of MX. This is particularly evident since long-term animal studies have shown carcinogenic and tumour promoting activity of MX (2,3). In the present study, we conducted transformation assays on MX using BALB/c 3T3 cells to give

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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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ELSEVIER

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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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), dimethyl-nitrosamine (DMN), p-dimethylaminoazobenzene (DAB), and di(2-ethylhexyl)phthalate (DEHP)—and two mutagenic chemicals—kojic acid and methylmethanesulfonate (MMS).

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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.

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
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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 × 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 hepatocel-

lular 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

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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 Micro-Flow®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 Micro-Flow®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
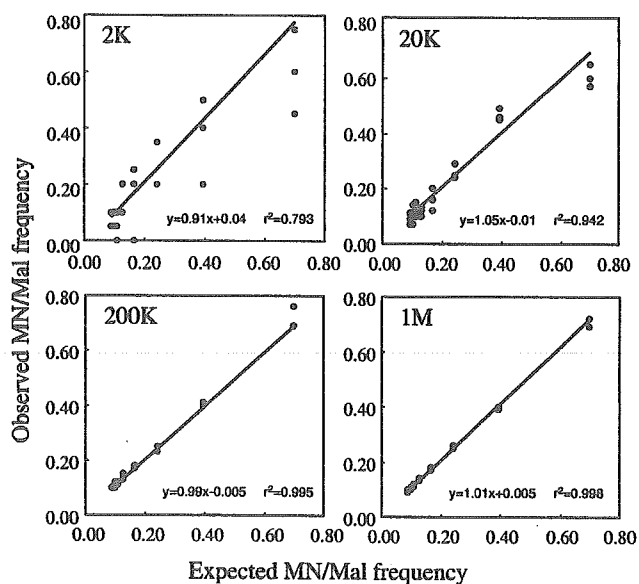

Fig. 1. Scattergram of expected versus observed MN/Mal frequencies. Each of three replicate measurements is plotted for these specimens. Best-fit linear lines are graphed, with associated equations. r^2 values document the degree of reproducibility observed.

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.

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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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