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# Development of a repeated-dose liver micronucleus assay using adult rats (II): Further investigation of 1,2-dimethylhydrazine and 2,6-diaminotoluene

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ABSTRACT

Detecting genotoxicity in the liver is considered an effective approach for predicting hepatocarcinogenicity, as many genotoxic chemicals *in vivo* may act as hepatocarcinogens in rodents. Here, a genotoxic rodent hepatocarcinogen, 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), and a genotoxic (Ames positive) noncarcinogen, 2,6-diaminotolunene (2,6-DAT), were administered orally to rats for up to 28 days, and liver samples were then examined in a repeated-dose liver micronucleus (MN) assay, and additionally tested in the bone marrow (BM) MN assay concurrently. We recently established a simple method to isolate hepatocytes without *in situ* liver perfusion procedures, and applied this method in the liver MN assay. As a result, 1,2-DMH increased the proportion of micronucleated hepatocytes in both a dose- and duration-dependent manner at relatively low-dose levels that are routinely used in repeated-dose toxicity studies. In contrast to 1,2-DMH, 2,6-DAT did not have a detectable effect. In addition to these two chemicals, two genotoxic rodent hepatocarcinogens, diethylnitrosamine and 2,4-diaminotoluene, which gave positive responses in the liver MN assay in our previous investigation [Narumi et al., Mutat. Res. 747 (2012) 234-239], were subjected to the BM MN assay and histopathological evaluation. All four test chemicals gave negative responses in the BM MN assay. Furthermore, the three hepatocarcinogens displayed hepatotoxicity, including hepatocellular hypertrophy and anisokaryosis, but no abnormal findings were observed in the liver of rats treated with 2,6-DAT. Taken together, the present results indicate that the liver MN assay is effective for predicting hepatocarcinogenicity and may be integrated into repeated-dose toxicity studies without disturbing routine examinations, such as histopathology. Furthermore, with repeat-dose treatment protocols, our findings indicate that the liver MN assay is superior to the BM MN assay for detecting genotoxic or carcinogenic chemicals in rats.



## 1. Introduction

Most previous micronucleus (MN) assays in the liver have been conducted using partial hepatectomy (PH) [1,2], mitogenic treatment [3,4], or in juvenile rats [5–8]. These studies indicate that the liver MN assay may be useful for the prediction of chemical hepatocarcinogenesis, even using chemicals that are insensitive to rodent bone marrow (BM) MN assays. Hepatocarcinogens are activated in the liver to yield genotoxic metabolites, which often do not reach BM cells due to their short lifetime [9]. In fact, several rodent hepatocarcinogens, including nitro-aromatic amines, alkylnitrosamines, and azo-derived chemicals, give negative results in BM MN assays [1]. Although many carcinogenic chemicals have been identified, tumor induction most frequently occurs in the liver [10]. Thus, it is anticipated that the MN-inducing potential of genotoxic hepatocarcinogens can be detected using liver MN assays [1,11,12].

Recently, participating members of the European Center for the Validation of Alternative Methods (ECVAM) Workshop agreed that the integration of *in vivo* genotoxicity tests, such as the BM MN assay, into repeated-dose toxicity studies presents one approach for reducing the number of animals used in experimentation [13]. Furthermore, to reduce the number of experimental animals, the International Conference on Harmonization (ICH) guidance on genotoxicity testing has recently been revised to recommend integration of *in vivo* genotoxicity studies, such as the BM MN assay, into general toxicity studies routinely conducted at the pre-clinical

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stage of pharmaceutical development [14]. However, the liver MN assays are typically performed using *in situ* liver perfusion *via* the portal vein or vena cava to isolate hepatocytes. Liver perfusion precludes conducting other tests that are commonly performed as part of repeat-dose general toxicology studies, such as histopathological examination.

To overcome the above-described limitations of *in situ* liver perfusion, we have recently established a remarkably practical and simple method to isolate hepatocytes from animals for the evaluation of liver MN inducibility [15]. Using this method, we reported that the rodent hepatocarcinogens diethylnitrosamine (DEN) and 2,4-diaminotoluene (2,4-DAT) significantly induced MN in the rat liver with repeated treatment regimens at least 14 days after dosing [15]. The establishment of this hepatocyte isolation method, which does not require perfusion of the entire liver, is expected to facilitate integration of the liver MN assay into general toxicity studies.

In the present study, we investigated the effectiveness of the liver MN assay conducted as part of a repeated-dose treatment protocol in order to confirm if this assay could be integrated into general toxicity studies. A genotoxic rodent hepatocarcinogen, 1,2-dimethylhydrazine dihydrochloride (1,2-DMH), and a genotoxic noncarcinogen, 2,6-diaminotoluene (2,6-DAT), were repeatedly administered to rats for up to 28 days, and MN induction in the liver and BM were then concurrently evaluated. In addition, samples from the identical animals used in the MN assays were subjected to histopathological examination.

In our previous report [15], DEN and 2,4-DAT were examined using only the liver MN assay; however, BM cells had been also collected from the identical animals. Furthermore, liver tissues other than those used for the liver MN assay had been fixed with formalin for later histopathological examination. Here, to further evaluate liver responses to hepatocarcinogens and noncarcinogens, the results of the MN assays and histopathology were compared between three hepatocarcinogens (1,2-DMH, DEN, and 2,4-DAT) and a noncarcinogen (2,6-DAT). Thus, this is a complementary paper to our previous report [15].

## 2. Materials and methods

### 2.1. Animals

Five-week-old male rats [Crl:CD(SD)] were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The animals were housed in an air-conditioned room maintained at 22 ± 3 °C and 55% ± 20% relative humidity under a 12-h light/dark cycle and allowed free access to a commercial pellet diet and tap water. The rats were quarantined and acclimated for a period of one week prior to the start of drug administration. The animal experiments were approved by the Institutional Animal Care and Use Committee of the test facility prior to conducting the experiments.

### 2.2. Chemicals

1,2-DMH (hydrochloride salt, CAS No. 306-37-6, >98% purity) and DEN (CAS No. 55-18-5, >99% purity) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and 2,6-DAT (CAS No. 823-40-5, >98% purity) and 2,4-DAT (CAS No. 95-80-7, >95% purity) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals were dissolved in physiological saline at appropriate concentrations to prepare dose formulations for administration to animals.

These chemicals were selected for reasons as follows: DEN and 2,4-DAT were used in our previous report and evaluated for their ability to induce MN in the liver of adult rats [15]; 1,2-DMH was expected to be positive in the liver MN assay in this study according to the previous report using young rats [8] although this chemical was reported to be negative in the BM MN assay in the 28-day repeated dose study [16]; and 2,6-DAT is structural isomer of 2,4-DAT and is classified as noncarcinogen [17], and furthermore this chemical was expected to be negative in the liver MN assay in this study.

### 2.3. Dose levels and treatment

Two dose levels were used for the administration of 1,2-DMH to be consistent with our previous study that used two dose levels of DEN and 2,4-DAT [15]. As 10 mg/kg/day was reported to be the maximum tolerated dose (MTD) of 1,2-DMH during a 28-day experiment [16], the high-dose level was set at 10 mg/kg/day, and the low-dose level was set at 5 mg/kg/day. The high dose level was expected to be positive in the present study based on the results of a previous report on MN induction in juvenile rats [8]. 2,6-DAT is known to be noncarcinogenic in rodents [17], and this chemical was expected to give a negative response in this investigation. Therefore, three dose levels in total were used for 2,6-DAT. The high-dose level was set at 100 mg/kg/day, which was equivalent to 40% of the level used in a previous report [18] in which rats were given a single oral gavage, and this dose level was anticipated to be the MTD for 28 days. The two lower doses were set at 50% and 25% of the high-dose level. For DEN and 2,4-DAT, the high dose levels were set at 12.5 and 50 mg/kg/day, respectively, as these levels were used in our previous investigation [15], and the low dose levels were set at 50% of the high dose level. Five animals were randomly assigned to each treatment group except for the 5- and 14-day experiments of 1,2-DMH, in which 3 animals were allocated to each group. The high dose level of each test chemical was administered once daily for 5, 14 or 28 days by oral gavage, and the lower dose level(s) of each chemical was administered up to 28 days. As the negative control, physiological saline used as the vehicle for preparation of the test formulations was administered in the same way as the test chemicals. All dose volumes were 10 mL/kg/day.

### 2.4. Liver MN assay

On the day after the last administration of the test chemicals for each time point (5, 14, and 28 days), the animals were euthanized by exsanguination under deep anesthesia without overnight fasting. The livers were excised, and the lateral left lobe of the liver was then removed. An approximately 1 g portion of the lobe was sliced into several pieces of 0.5- to 1-mm thicknesses. The sliced tissues were washed with cold Hank's balanced salt solution (HBSS) and treated with a digestion solution containing collagenase (Wako Pure Chemical Industries, Ltd., Osaka Japan) to isolate hepatocytes (HEPs) using the methods established by Narumi et al. [15]. Isolated HEPs were fixed with 10% neutral buffered formalin, and the resultant suspensions of HEPs were stored at room temperature until needed for microscopic observation.

Immediately prior to microscopic observation, each HEPs suspension was mixed and stained with an equal volume of staining solution consisting of acridine orange (AO, 500 μg/mL) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 10 μg/mL). The resultant mixture was dropped onto a clean glass slide and covered with a cover slip (24 mm × 50 mm) to prepare slide specimens. The specimens were observed under a fluorescent microscope at 400× magnification with U-excitation (Ultraviolet rays excitation, wave length: 330–385 nm), and the number of micronucleated HEPs (MNHEPs) per 2000 parenchymal HEPs was counted for each animal according to the previous reports [5–8].

### 2.5. BM MN assay

After the removal of livers as described in the liver MN assay, femurs were removed from the same animals. BM cells were collected from the femurs according to a method reported by Kawabata et al. [19], and the resultant BM cell suspensions were stored at room temperature until used for microscopic observation.

Immediately prior to microscopic observation, each BM-sample was mixed and stained with an equal volume of an AO (500 μg/mL) solution. The resultant mixture was dropped onto a clean glass slide and covered with a cover slip (24 mm × 50 mm) to prepare slide specimens. The specimens were observed under a fluorescent microscope at 600× magnification with B-excitation (Blue light excitation, wave length: 420–490 nm), and the number of micronucleated immature erythrocytes (MNIMEs) per 2000 erythrocytes was counted for each animal. As a parameter of hematopoietic function in BM, the proportion of immature erythrocytes (IMEs) among 1000 erythrocytes was also calculated for each animal.

### 2.6. Histopathological examination

Upon euthanasia, the residual liver tissue of the left lateral lobe after the isolation of HEPs was fixed with 10% phosphate buffered formalin, embedded in paraffin, and then stained with hematoxylin and eosin (H.E.) according to the standard method. Histopathological examination was performed under a light microscope.

### 2.7. Statistical analysis

Differences in the incidences of MNHEPs and MNIMEs between the test and the vehicle control groups were analyzed using the conditional binomial test reported by Kastenbaum and Bowman [20]. To analyze the percentage of IME ratios between groups, Dunnett's multiple comparison test was applied for the 28-day experiment since two or three groups were treated with each test chemical. For the 5- and 14-day experiments, Student's *t*-test was applied since there was only one group of each test chemical.

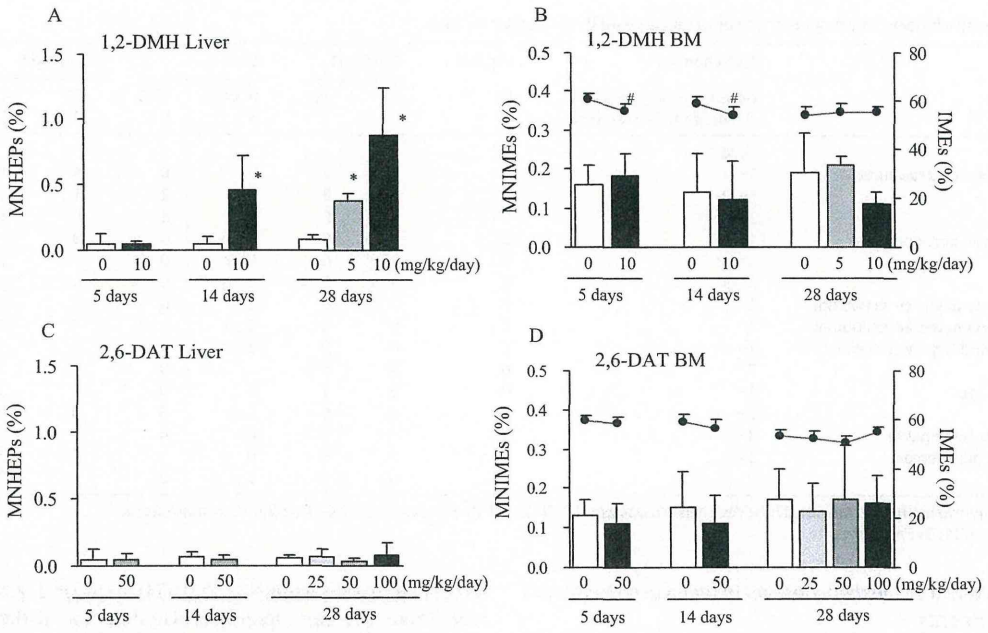


**Fig. 1.** Proportion of MNHEPs (%) in rat livers (bar graphs, left) and ratio of MNIMEs (bar graphs, right) and IMEs-ratio (line graphs, right) in rat BM treated for 5, 14, or 28 days with 1,2-DMH (A, B) or 2,6-DAT (C, D). Values are presented as the mean and SD ($n = 5$ or 3). $^{*}p < 0.01$, Kastenbaum and Bowman test, $^{\#}p < 0.05$, Student's *t*-test.

## 3. Results

### 3.1. Liver and BM MN assays

Fig. 1 shows the results of the liver and BM MN assays in rats treated with 1,2-DMH and 2,6-DAT, and Fig. 2 shows the results of the BM MN assay with DEN and 2,4-DAT administered to rats used in the previously reported liver MN assay [15]. In the liver MN assay, the mean MNHEPs % in the vehicle control groups were 0.05 ± 0.07%, 0.05 ± 0.05%, and 0.08 ± 0.03% for 5, 14 and 28 days in 1,2-DMH experiment, respectively. For 2,6-DAT in the same way, the mean MNHEPs % in the vehicle control groups were 0.05 ± 0.07%, 0.07 ± 0.03%, and 0.06 ± 0.02% for 5, 14 and 28 days, respectively. 1,2-DMH significantly increased the proportion of MNHEPs after repeated dosing for 14 days or longer (mean MNHEPs %: 0.45% at 12.5 mg/kg/day for 14 days, 0.37% at 6.25 mg/kg/day for 28 days and 0.87% at 12.5 mg/kg/day for 28 days), but had no effect in the 5-day experiment (mean MNHEPs %: 0.05% at 6.25 mg/kg/day, Fig. 1A). In the 28-day experiment, a dose-dependent increase in the incidence of MNHEPs was observed. Additionally, the incidence of MNHEPs tended to display a duration-dependent increase. In contrast to 1,2-DMH, treatment with 2,6-DAT did not result in significant increases in the incidence of MNHEPs in the liver at any examined time point; the mean MNHEPs % ranged from 0.03% to 0.08% (Fig. 1C).

In the BM MN assay, no significant increases in the proportion of MNIMEs were detected during the 28 days of treatment with either 1,2-DMH or 2,6-DAT (Fig. 1B and D, respectively). In the 28-day experiment with 2,4-DAT, one animal each was found dead on day 15 and 24 after the initial administration at 50 mg/kg/day, while no mortality was observed in the other groups administered 2,4-DAT. All surviving animals were used for the evaluation of the BM MN assay for 2,4-DAT.

For both 2,4-DAT and DEN, no significant increases in the proportion of MNIMEs in the BM MN assay were observed at any time point up to 28 days (Fig. 2). In the 5-day experiment with DEN (Fig. 2A) and the 5- and 14-day experiments with 1,2-DMH (Fig. 1B), significant decreases in the IMEs ratio were detected. However, no marked differences were observed in the IMEs ratios for the 28-day experiments for any test chemical compared to the vehicle control group.

### 3.2. Histopathology

Table 1 presents the test chemical-related histopathological findings in the liver and Fig. 3 shows the typical hepatotoxicity

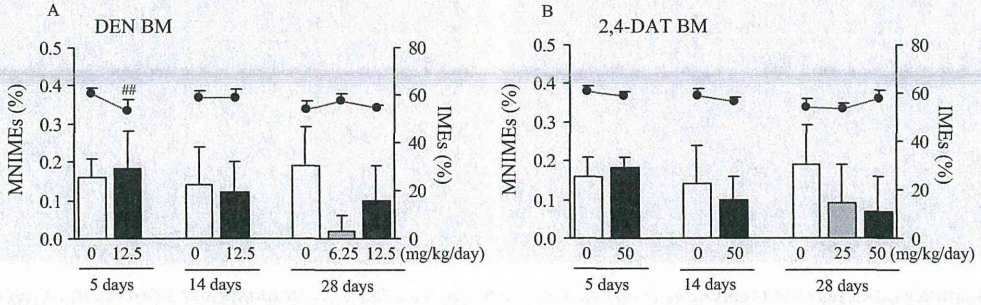


**Fig. 2.** Proportion of MNIMEs (bar graphs) and IMEs ratios (line graphs) in rat BM treated for 5, 14, or 28 days with DEN (A) or 2,4-DAT (B). Values are presented as the mean and SD ($n = 5$). $^{\#\#}p < 0.01$, Student's *t*-test.

**Table 1**
Test chemical-related histopathological findings in the liver of rats repeatedly treated for 28 days.

| Finding | Test chemical | Vehicle | 1,2-DMH | | DEN | | 2,4-DAT | | 2,6-DAT |
|---|---|---|---|---|---|---|---|---|---|
| | Dose level (mg/kg/day) | 0 | 5 | 10 | 6.25 | 12.5 | 25 | 50 | 100 |
| | No. of animals examined | 5 | 5 | 5 | 5 | 5 | 5 | 3 | 5 |
| | Grade | | | | | | | | |
| Hypertrophy, hepatocyte, centrilobular | 1+ | 0 | 0 | 2 | 0 | 0 | 4 | 1 | 0 |
| | 2+ | 0 | 0 | 3 | 0 | 2 | 1 | 1 | 0 |
| | 3+ | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 0 |
| Hypertrophy, hepatocyte, perilobular | 1+ | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | 2+ | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| | 3+ | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Acidophilic change, hepatocyte, centrilobular | 1+ | 0 | 5 | 0 | 2 | 0 | 0 | 0 | 0 |
| Single cell necrosis, hepatocyte, centrilobular | 1+ | 0 | 5 | 5 | 4 | 5 | 3 | 3 | 0 |
| Cell infiltration, inflammatory, centrilobular | 1+ | 0 | 0 | 0 | 2 | 2 | 4 | 0 | 0 |
| | 2+ | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Anisokaryosis, hepatocyte | 1+ | 0 | 0 | 3 | 0 | 0 | 1 | 3 | 0 |
| | 2+ | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 |
| Increase, mitotic figure, hepatocyte | 1+ | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 |
| Proliferation, oval cell, perilobular | 1+ | 0 | 0 | 0 | 0 | 3 | 0 | 1 | 0 |
| | 2+ | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |

1,2-DMH: 1,2-dimethyl hydrazine hydrochloride; DEN: diethylnitrosamine; 2,4-DAT: 2,4-diaminotoluene; 2,6-DAT: 2,6-diaminotoluene.
Grade—1+: minimal; 2+: mild; 3+: moderate.

observed in rats treated at the high-dose level for each test chemical in the 28-day experiments.

Centrilobular hypertrophy of hepatocytes was observed in all of the surviving animals treated at the high dose levels of the three hepatocarcinogens, 1,2-DMH, DEN, and 2,4-DAT. Notably, centrilobular hypertrophy was also detected at the low dose level of 2,4-DAT, which additionally induced perilobular hypertrophy of hepatocytes. For all chemicals, the observed hepatocellular

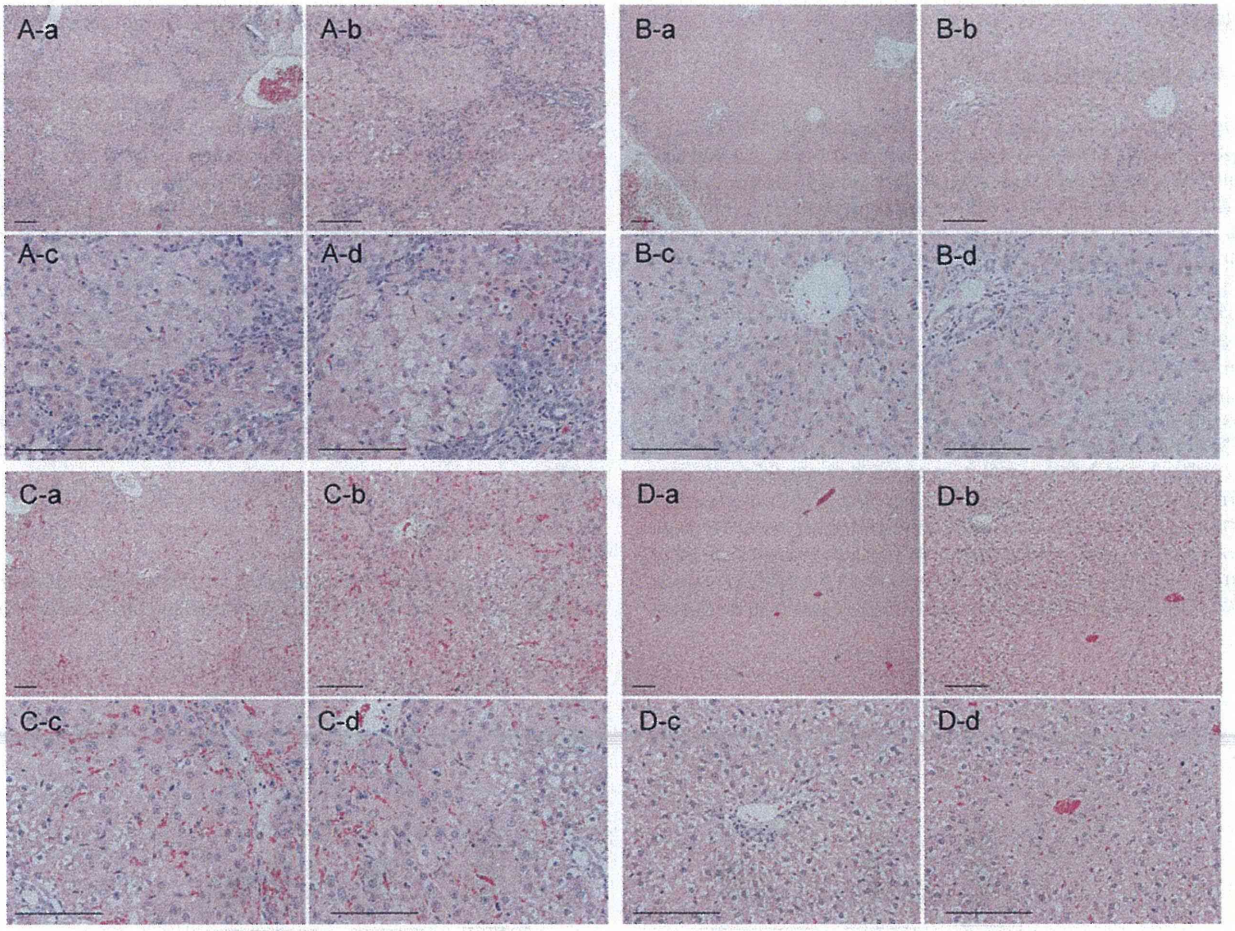


**Fig. 3.** H.E. stained histopathological images of rat livers treated for 28 days with 12.5 mg/kg/day of DEN (A), 50 mg/kg/day of 2,4-DAT (B), 10 mg/kg/day of 1,2-DMH (C), or 100 mg/kg/day of 2,6-DAT (D). Original magnifications 10× (a), 20× (b), and 40× (c, centrilobular area; d, perilobular area). Hepatotoxicity was observed in the livers of rats administered DEN, 2,4-DAT, and 1,2-DMH. Bar = 100 μm.

**Table 2**
Mean body weight changes of rats repeatedly treated with 1,2-DMH and 2,6-DAT for 28 days.

| Test chemical (dose, mg/kg/day) | Day 1 | | Day 6 | | Day 15 | | Day 29 | |
|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Ratio[a] | Mean ± SD | Ratio[a] | Mean ± SD | Ratio[a] | Mean ± SD | Ratio[a] |
| Vehicle | 229.4 ± 10.1 | 1.00 | 271.8 ± 13.8 | 1.00 | 343.6 ± 22.2 | 1.00 | 431.2 ± 33.5 | 1.00 |
| 1,2-DMH (5) | 232.0 ± 13.3 | 1.01 | 264.6 ± 13.0 | 0.97 | 330.0 ± 15.2 | 0.96 | 395.8 ± 30.2 | 0.92 |
| 1,2-DMH (10) | 229.6 ± 8.2 | 1.00 | 241.0 ± 11.2 | 0.89 | 284.9 ± 24.6 | 0.83 | 341.0 ± 20.2 | 0.79 |
| Vehicle | 213.3 ± 7.0 | 1.00 | 261.1 ± 8.2 | 1.00 | 333.8 ± 13.5 | 1.00 | 426.8 ± 21.1 | 1.00 |
| 2,6-DAT (25) | 213.0 ± 10.0 | 1.00 | 243.4 ± 11.7 | 0.93 | 307.2 ± 17.7 | 0.92 | 374.8 ± 30.4 | 0.88 |
| 2,6-DAT (50) | 214.1 ± 7.1 | 1.00 | 243.5 ± 9.5 | 0.93 | 308.0 ± 15.2 | 0.92 | 382.0 ± 25.0 | 0.90 |
| 2,6-DAT (100) | 215.8 ± 11.1 | 1.01 | 234.2 ± 18.9 | 0.90 | 292.6 ± 28.5 | 0.88 | 359.2 ± 36.3 | 0.84 |

[a] Ratio to the vehicle control.

hypertrophies involved acidophilic changes. At the low dose levels of 1,2-DMH and DEN, centrilobular acidophilic changes were induced without hepatocellular hypertrophy. Centrilobular single cell necrosis of hepatocytes was observed at all dose levels of the three hepatocarcinogens, and hepatocellular anisokaryosis was detected in the high dose groups at a minimum. Additionally, increased mitotic figures and perilobular oval cell proliferation were observed in animals administered the high dose of DEN. Oval cell proliferation was also found in one animal at the high dose level of 2,4-DAT. In contrast, no abnormal findings were observed in animals treated with 2,6-DAT or vehicle control.

## 4. Discussion

The present study was conducted to further investigate the effectiveness of the liver MN assay for the detection of genotoxic hepatocarcinogens. Moreover, one of the aims of our present and previous studies [15] was to demonstrate that the liver MN assay is suitable for integration into general toxicity studies. Here, the effects of the genotoxic hepatocarcinogens, DEN, 2,4-DAT, and 1,2-DMH, and the noncarcinogen 2,6-DAT administered to rats by repeated oral treatment for up to 28 days were examined in a liver MN assay that incorporated an established hepatocyte isolation method [15] as an alternative to *in situ* liver perfusion.

In the liver MN assay, 1,2-DMH gave a positive response that exhibited a dose- and duration-dependency (Fig. 1A) which was similar to that observed in our previous report examining DEN and 2,4-DAT [15]. However, the noncarcinogen 2,6-DAT did not increase the incidence of MN in the liver MN assay (Fig. 1C). Together, our present and previous [15] results for these four chemicals suggest that the liver MN assay for samples obtained from animals administered a repeated dose regimen is an effective method to detect genotoxic rodent hepatocarcinogens.

For the BM MN assay, a single dosing of 1,2-DMH at 200 mg/kg gave a positive result [21], whereas a repeated dose regimen for 28 days was reported to give a negative result [16]. It was also found that short-term DEN and 2,4-DAT treatments were associated with negative or weak responses in BM MN assays for rats and mice [9,22]. The results of these previous reports [9,16,21] are consistent with the present BM MN assay results for these three hepatocarcinogens (Figs. 1B and 2A, B). Together, the results of our present and previous [15] studies suggest that genotoxic hepatocarcinogenicity, which is typically undetectable by the standard BM MN assay, can be assessed by the liver MN assay. In addition, as the liver is the main target organ for the carcinogenicity of DEN and 2,4-DAT [23,24], whereas 1,2-DMH targets multiple organs in addition to the liver in rats and mice [25], the liver MN assay may be highly organ specific for the prediction of hepatocarcinogenic potential of test chemicals.

For 2,6-DAT, the high dose level (100 mg/kg/day) used in the present study is equivalent to 40–67% of the levels used in previous short-term assays [18,26]. In addition, the mean body weight of animals administered this dose was 16% lower than the vehicle control group at the termination of the 28-day experiment (Table 2). Integration of the liver MN assay into the general toxicity study is one of the aims of this investigation. According to our plan, the pattern of body weight changes in rats treated with 2,6-DAT was comparable to those produced by 1,2-DMH (Table 2) and other two hepatocarcinogens [15], although no statistical analyses were conducted in this investigation. Thus, the high dose level selected for 2,6-DAT was considered to be appropriate for a 28-day repeated dose general toxicity study. However, the top dose of 2,6-DAT may not have reached the MTD for the genotoxicity study because there were no histopathological findings (Table 1 and Fig. 3D), and the effect on the body weight gain was not severe (Table 2). We supposed that the doses at 200–250 mg/kg of 2,6-DAT could not be used for the 28-day treatment since these dose levels are around a half of $LD_{50}$ [18]. Thus, we did not conduct the repeated administration of 2,6-DAT at around these doses. Dose selection may be a problem which confronts us in the future in order to truly integrate a liver MN assay into a repeated dose general toxicity study. In the present study, we did not analyze the parameters of either hematology or blood chemistry. These data also should be analyzed to determine the MTD for the integration of the assay into the general toxicity study. Furthermore, in the 5- and 14-day experiments for 2,6-DAT, a dose of 50 mg/kg/day was selected to allow comparison with the identical dose used for 2,4-DAT, its structural isomer. It may have been possible to administer rats a higher dose of 2,6-DAT, at least 100 mg/kg/day in the 5- and 14-day experiments. However, considering the integration of the liver MN assay into a general toxicity studies at the drug developmental stage, a repeated dose regimen for 28 days or longer would be preferable.

The aromatic amines 2,4-DAT and 2,6-DAT are structural isomers and are often compared with regard to their carcinogenic potential. Both 2,4-DAT and 2,6-DAT are mutagenic in *Salmonella typhimurium* strains [27] and *in vitro* chromosome aberration assays [22,28], and give negative or weakly positive results in the BM MN assay [22]. Despite the fact these chemicals exhibited the same results in these genotoxicity studies, only 2,4-DAT is carcinogenic in rats and mice [24,29]. Therefore, the carcinogenic potency of these two isomers cannot be distinguished by the genotoxicity studies commonly required by regulatory paradigms. In contrast, several *in vivo* assays other than the BM MN assay are reported to give positive responses for only 2,4-DAT [17,26,27,30,31]. For example, these two isomers were examined using short-term assays involving the unscheduled DNA synthesis test [26] and *lacZ* transgenic Muta™Mouse [31], with the results being compared to those of the $^{32}$P-postlabelling technique [17], gene expression profiling technology [30], and *gpt* delta transgenic rats [27] following repeated dosing for approximately one month or longer. Although the results of these previous studies and those for 2,4-DAT [15] and 2,6-DAT in this study (Fig. 1C, D) are all consistent with the reported carcinogenicity of these isomers in rats and mice, our liver MN assay is considered to be easier and less expensive to conduct than these other short-term assays and methods of similar duration.

**Table 3**
Summary results of our investigations on the liver and bone marrow micronucleus assay.

| Test chemical | Liver MN | | | BM MN | | | Histopathological findings[a] | Carcinogenic target organ in rats | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sampling time (day) | 5 | 14 | 28 | 5 | 14 | 28 | | Liver | Others | Reference |
| 1,2-DMH | – | + | + | – | – | – | + | Positive | Positive multi-organs | [25] |
| 2,6-DAT | – | – | – | – | – | – | – | Negative | Negative | [29] |
| DEN | (+) | (+) | (+) | – | – | – | + | Positive | Positive multi-organs | [23] |
| 2,4-DAT | (–) | (+) | (+) | – | – | – | + | Positive | Positive mammary gland | [24] |

+: increased or observed in our investigation, –: not increased or not observed in our investigation.
Parentheses indicate the result of the previous study by Narumi et al. [15].
[a] Findings related to hepatocyte proliferation.

The hepatotoxicity of the test chemicals was evaluated in the present study by histopathological examination (Table 1 and Fig. 3), which is commonly conducted in general toxicity studies and which we were able to perform here in conjunction with the liver MN assay, as *in situ* liver perfusion is not necessary for our liver MN assay methods. The three examined hepatocarcinogens displayed hepatocellular hypertrophy, single cell necrosis, and/or acidophilic changes, which represent early stage histopathological findings of hepatotoxicity, while the observed inflammatory cell infiltration, anisokaryosis, increase of hepatocellular mitotic figures, and/or proliferation of oval cells are speculated to have occurred in response to the early stage changes (Table 1 and Fig. 3). It is commonly known that hepatocyte hypertrophy is found in the liver of rodents treated with a hepatocarcinogen. It is considered that single cell necrosis is followed by cell proliferation, and hepatocellular anisokaryosis and an increase in mitotic figures indicate elevated proliferative activity of hepatocytes. Whereas liver oval cells are considered to proliferate and differentiate only under conditions in which hepatocyte proliferation is blocked or delayed [32], the oval cells are expanded by administration of 2-acetylaminofluorene plus PH model [33]. It is considered that the histopathological findings observed in rats treated with these three carcinogens are closely related to hepatocyte proliferation. In contrast to these carcinogens, 2,6-DAT gave no detectable findings in the histopathological examination of liver tissue (Table 1 and Fig. 3). Table 3 shows summary results of our present and previous [15] investigation of the liver and BM MN assays and histopathological examination. The observed histopathological changes correlate with the results of the liver MN assay in our present and previous [15] investigations.

When the MN assay is conducted using adult rat liver tissue, measurement of the cell division rate is important, as the liver undergoes low cell division. It is also critical to evaluate whether cell division was suppressed when a negative result is obtained in the liver MN assay. For the BM MN assay results shown in Figs. 1 and 2, the IME-ratio is a useful indicator of cell division suppression. In the liver, internal cell-markers, such as Ki67 and PCNA, or BrdU-incorporation are effective indicators of cell proliferation [34]; however, no appropriate indicators of cell division have been identified, with the exception of counting mitotic figures, as DNA synthesis in the liver occasionally occurs in the absence of cell division [35]. Furthermore, evaluating mitotic figures in the livers of adult rats is considered to be inappropriate because rats over the age of 6 weeks have markedly low cell division activity [15]. Supporting this finding, the percentage of mitotic figures detected in the individual rats examined here ranged from 0.00% to 0.10% for all groups administered 1,2-DMH or 2,6-DAT. Moreover, it is practically and routinely considered that scoring 2000 cells is a marginal number to calculate the incidence of MNHEPs; therefore, the data of mitotic figure counts in hepatocytes were not reported here. In the liver MN assay using PH [36,37], the number of main nuclei in the HPEs (*i.e.* mononucleated, binucleated or multinucleated [3 nuclei or more]) may be useful as a dividing index of HEPs, however, identifying an appropriate indicator of cell division for the liver MN assay remains a future challenge, and so has been for the MN assay in human buccal cells [38]. When a negative result is obtained in the liver MN assay, histopathological examination might provide meaningful information, as was the present case for 2,6-DAT, which did not show any abnormal findings (Fig. 3). We speculate that an absence of abnormal findings in the histopathological examination indicates that the cell division rate is low, but steady, in the adult rat liver, when the liver MN assay also gives a negative result.

It has been speculated that MNHEPs accumulate following repeated dosing with test chemicals due to the long lifespan of HEPs [32]. The proportion of MNHEPs observed for 1,2-DMH (Fig. 1), DEN and 2,4-DAT [15] increased in a dosage- and duration-dependent manner, a finding that supports the speculation that MNHEPs remain in the liver where they accumulate. However, based on the present histopathological examination results, it cannot be excluded that the incidence of MNHEPs was increased by hepatocellular multiplication after treatment with the three test hepatocarcinogens. In the future, it is necessary to determine how this finding relates to the results of the liver MN assay.

Although it has been reported that the rat BM MN assay can be integrated into general toxicity studies [16], a chemical, such as 1,2-DMH, which has to be administered at lower levels during repeated dosing than those used for single or double dosing cannot be detected by the BM MN assay with the repeated dose regimen. To detect MN in the BM according to a previous report [16], a greater number of testing points were necessary in the present study, such as examination of the proportion of MN in peripheral blood and spleen histopathology approximately 4 days after the initial dosing. In the present and previous [15] studies, however, the genotoxic hepatocarcinogens 1,2-DMH, DEN, and 2,4-DAT significantly induced MNHEPs at lower dose levels than those used in the previous reports [5,8,21]. Based on our present findings and the speculation concerning MNHEPs accumulation in the liver as described above, the liver MN assay is considered to be more appropriate for a repeated dose study than the BM MN assay.

An important trend in current toxicology is the 3R-principles, which stands for the replacement, reduction, and refinement of the use of experimental animals. Integration of the liver MN assay into general toxicity studies would help realize one of the 3R-principles, namely a reduction in the number of animals required for the ECVAM [13] and the revised ICH guidance [14]. Furthermore, we expect that the reduction of the number of experimental animals will contribute to a reduction in the cost of the animal experiments; labor, time, animals and other materials used for the experiments. Based on the results of our present and previous studies [15], the liver MN assay can be integrated into 28-day repeated dose toxicity studies that are typically conducted during the toxicological evaluation of pharmaceutical drugs and agrochemicals. Such integration may further facilitate a reduction in the number of experimental animals required for regulatory toxicology.

In conclusion, integrating the liver MN assay into repeated dose toxicity studies is useful and effective for evaluating *in vivo* clastogenicity and predicting the hepatocarcinogenicity of test chemicals.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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FOOD SAFETY


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Review (Invited)

# Identification and Evaluation of Potentially Genotoxic Agricultural and Food-related Chemicals

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The Food Safety Commission (FSC) was founded in 2003 to conduct the risk assessment of chemicals in food and food products and also residues of agricultural chemicals. Genotoxicity assessment is one component of the overall risk assessment process. Historically, genotoxicity assessment has been limited mainly to qualitative hazard identification. We are proposing a strategy for when the chemical is classified as a genotoxic carcinogen and the acceptable daily intake (ADI) cannot be set because a worldwide consensus has not been obtained on the existence of threshold for DNA direct-acting genotoxicity. To evaluate the mechanism(s) of carcinogenicity, it is important to make judgment whether genotoxicity, especially genotoxicity/mutagenicity resulting from direct reaction with DNA, is a key event or not in the carcinogenic process. Here, we focus on the residues of agricultural chemicals and discuss the strategy of how to evaluate and interpret genotoxicity, and provide guidance that we can use at the site of assessment. This paper presents the authors' personal opinion and it does not necessarily represent the official opinion of the FSC. There are four independent expert working groups in the Expert Committee for evaluation of agricultural chemicals and the authors hope this paper will help to make evaluation fair and transparent across the working groups. Of course, other strategies to evaluate genotoxicity of food and food related chemicals, including residues of agricultural chemicals may also exist, and they should also be appreciated. The goal is scientifically sound, transparent, and fair evaluation and interpretation of genotoxicity, as an integral part of the risk assessment.

**Key words:** pesticide and agricultural chemicals, risk assessment on mutagenicity, evaluation and interpretation

## Introduction

The Food Safety Commission (FSC) was founded in 2003 to make risk assessment of food and related chemicals, independent from risk management. Before that time, mainly the Japanese Ministry of Health, Labour and Welfare (MHLW: former Ministry of Health and Welfare) conducted risk assessment in conjunction with risk management of

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chemicals and covered almost all areas including pharmaceutical drugs, agricultural chemicals, and food and food related chemicals. A principal aim of the FSC was to make risk assessment independent from risk management.

Evaluation and interpretation of chemical safety is generally based on the toxicological tests to identify hazards, including carcinogenicity, mutagenicity, reproductive toxicity, neurotoxicity, and other specified toxicological endpoints. Among them, carcinogenicity, mutagenicity, and reproductive toxicity are regarded as important hazards for assessing human health assessment and called "CMR effects"[1]. Generally, CMR effects are well evaluated especially in the case of food and related chemicals, *e.g.*, food additives, pesticides and veterinary drugs[2], which are consumed every day unlike pharmaceutical drugs which are used intermittently in most cases.

Mutagenicity is usually defined as induction of a heritable alteration in DNA, usually gene mutation or chromosomal aberration. Mutagens may damage DNA directly or indirectly, or may modify the mitotic apparatus with a resulting infidelity of maintenance of the genome balance in cells. Such damage is often a key initial event of carcinogenicity and termed initiation, and also has the potential to cause heritable adverse events in subsequent generations. When the chemical of interest has not been evaluated for carcinogenicity, then mutagenicity can be used as a predictor of carcinogenic potential. When a chemical is carcinogenic in experimental animals, then assessment of mutagenic activity is an important component of evidence used to determine the mechanism of the carcinogenicity. If the chemical does not show any mutagenic potential, then carcinogenicity must be induced by a non-mutagenic mechanism—and non-mutagenic mechanisms are generally considered to exhibit a threshold exposure level below which carcinogenicity does not occur. Because mutagenesis that results from direct modification of DNA is generally considered to lack a threshold (*i.e.*, low exposures are considered to still carry some level of risk), determination of a mutagenic mode of action is a key element of cancer and reproductive risk assessment. The definition of a genotoxic carcinogen was proposed in the evaluation guideline of the food additive evaluation group of FSC as follows: "A genotoxic carcinogen is a chemical or its metabolite that reacts directly with DNA resulting in gene mutation or chromosomal aberration and the genotoxic effect(s) is considered to be the mechanism, at least a part of the mechanism, of carcinogenicity. It is necessary that genotoxicity is confirmed *in vivo*, preferably at the target organ of carcinogenicity."

Gene mutations and chromosomal aberrations are fixed lesions in the DNA that can be transmitted to future generations of cells or organisms. The initial event of mutation is the interaction between DNA and the molecule of interest. The interaction is based on a probability that depends on the densities of both molecules. This may be the reason why mutation does not have real threshold concentration below which the event does not occur. DNA damage, adduct formation, DNA repair in relation to the DNA damage, and sister-chromatid exchange may be prior events to the mutation and many times such events are included in the definition of genotoxicity.

Genotoxicity is assessed by many test systems designed to detect specific endpoints. Genotoxic activity can be classified by the endpoint and the test systems, *e.g.*, mutation, chromosomal aberration, DNA adducts, etc. *in vitro* or *in vivo*. *In vitro* assay systems include tests using microbial systems, for example Ames gene mutation (Salmonella/microsome) assay is the most well-known, and is widely used for initial assessment of chemical genotoxicity. The *in vitro* assay is more hazard identification oriented while the *in vivo* assay systems can provide information more relevant to assessment of human safety. Historically, the assessment of genotoxicity has been made qualitatively, just classification as positive or negative regardless of the potency of the effects, and used with the objective of hazard identification. Currently, the field is moving toward a more quantitative risk assessment that uses exposure-response relationships to determine the safety to humans based on the evaluation of exposure in relation to mutagenic potency.

## Role of FSC

The FSC prepares dossiers of the safety of food related chemicals based on animal and some *in vitro* studies, and determines the acceptable daily intake (ADI) by voluntary specialists from each field. Risk assessment is conducted by the process of hazard identification, dose-relationship analysis, exposure analysis, and risk characterization. The relationship among FSC, MHLW, and the Ministry of Agriculture, Forestry and Fisheries (MAFF) and other related Ministries is described at the following website: http://www.fsc.go.jp/english/aboutus/roleofthefoodsaftycommission_e1.html. The FSC makes the exposure analysis based on, for example, the residue levels of pesticides from the test field and by national investigations of food consumption. After the report of safety information and of the ADI of chemicals to the MHLW, which decides how to manage to use and report back on the intake of the chemical relative to the ADI based on the estimated human consumption, *e.g.*, for infants, adults, and aged people. Therefore, the FSC is able to understand the exposure levels of agricultural chemicals and make risk communications based on the risk characterization.

**Table 1.** Representative Test Classified by Endpoints

| | DNA damage | Gene mutation | Chromosomal aberration |
|---|---|---|---|
| *In vitro* | Rec assay<br>Umu assay<br>$^{32}$P-post labeling assay<br>Alkaline elution assay<br>Comet assay | Microbial reverse mutation assay (OECD TG 471)*<br>HPRT gene mutation assay using Chinese hamster cells (OECD TG476)<br>Mouse lymphoma TK assay (MLA, OECD TG is preparing) | Metaphase analysis using cell Line or primary culture (OECD TG473)<br>*In vitro* micronucleus assay (OECD TG478)<br>Mouse lymphoma TK assay (MLA, OECD TG is preparing) |
| *In vivo* | Unscheduled DNA sysnthesis Assay (OECD TG486)<br>Alkaline elution assay comet assay (OECD TG is preparing) | Transgenic animal mutation model (OECD TG488) | Bone marrow metaphase analysis (OECD TG475)<br>Rodent micronucleus assay (OECD TG474) |

* Assay with underline is a component of standard test battery in fields of pesticides, agricultural chemicals, and pharmaceuticals

There are four independent Expert Committees for evaluation of agricultural chemicals under umbrella of the Executive Committee, one of which purpose is to make balance among four Expert Committees. The authors hope this paper will be a guidance to help makings evaluation fair and transparent across the working groups.

Basically, the FSC evaluates the safety of material as a single chemical and not as a complex mixture, to which people usually are exposed. There remains a great need to assess complex mixtures or combinations of chemicals to which humans are exposed. To consider such multiple chemicals exposure, the concept of threshold is important to evaluate overall risk if each chemical is evaluated separately, because, if each chemical has no threshold genotoxic effects the effects should be accumulating,and may reach the virtual human risk. Another important and difficult issue is the reliability of extrapolation from unrealistically high concentrations/dose experiments to the actual human exposure level. Generally, laboratory assays for genotoxicity are conducted at quite high concentrations/dose levels because of technical considerations.

Evaluation of the safety of agricultural chemicals is not simple. People usually consume vegetables, for example, after careful washing and cooking (heating). Therefore, the agricultural chemical and probably metabolite residues are diminished during preparation. Thus, actual exposures are often overestimated. This provides an additional margin of safety between our actual consumption and the estimated exposure, in relation to the ADI.

## Genotoxicity Test Systems

To detect genotoxicity of chemicals many test methods have been developed and used in both research and regulatory science fields. There are two principal endpoints thought to be related directly to health risk—gene mutation and chromosomal aberration—among other supportive endpoints. The most important secondary endpoint is DNA damage that can lead to mutations or chromosomal aberrations. There is no assay system that detects all endpoints in one experiment. Therefore we use several assay systems to cover at least two major endpoints. This evaluation strategy is called a "battery" approach and has been accepted in the regulatory science field to avoid making false negative conclusions for the chemicals. **Table 1** shows representative assay systems classified by endpoint and test materials.

An important consideration is the choice of test systems. One major classification is *in vitro* vs. *in vivo* assays. Gene mutation was originally recognized and evaluated using plants by Mendel, and much basic mutation research has been conducted using microbial assays and plant cells. In the field of regulatory science, Bruce N. Ames developed the assay system to detect mutagenic chemicals using *Salmonella typhimurium* TA strain series in combination with an exogenous metabolic activation system to mimic mammalian metabolism (OECD TG471)[3]. These tester strains were modified to increase their sensitivity to detect either base-change gene mutation or frame-shift mutation. The so-called Ames assay is performed in Petri plates, and so we call this an *in vitro* assay although it uses a whole organism (bacterium). To detect another endpoint, chromosomal aberrations, cultured Chinese hamster cells are frequently used because of the small number of large chromosomes, which can be easily scored for chromosomal aberrations. Recently, the selection of target cells has become an important issue for regulatory science because it has been recognized that the species or origin and