We have already discussed the application of automated systems to scoring of the rodent micronucleus assay in Washington D.C. in 1999 at the 2nd IWGTP [3]. We summarized the discussion on this topic as follows: "In summary, although early versions of automated scoring techniques suffered from unacceptable levels of artifacts and/or were deficient with respect to distinguishing immature erythrocytes from mature erythrocytes, the modern systems described herein have overcome these limitations. These flow cytometry and image analysis techniques can be expected to replace microscopy-based scoring by providing reliable data in a more efficient manner. However, rigorous validation and adequate quality-control systems are necessary prerequisites to the use of automated scoring." At the 2nd IWGTP, we also discussed the validation criteria and drew the following conclusions:

"Considering the OECD general points, each system should also meet the following validation criteria:

1. Results obtained should be demonstrated to be comparable with those obtained by direct manual microscopic scoring of:
   (a) micronucleated immature erythrocyte frequency
   (b) micronucleated mature erythrocyte frequency, and
   (c) percentage of immature erythrocytes among total erythrocytes.

   This demonstration must be made for each tissue and species analyzed, and should verify that all cell types of interest (e.g., micronucleated immature and mature erythrocyte, immature and mature erythrocyte) are adequately identified and scored.
2. Micronuclei arising from both chromosome fragments and whole chromosomes should be demonstrated to be scored consistently.
3. Scoring should be shown to be consistent within and between experiments.
4. Preferably, laboratories should establish how known potential artifacts (platelets, basophilic granules, Heinz bodies, aggregated RNA, etc.) behave in their system.

At the 4th IWGT, we re-visited this topic and we focused on the flow cytometric method for the micronucleus assay. Image analysis is acknowledged to also be a useful automated methodology, but because image analysis is more closely related to manual microscopy and the image can be retrieved to the microscopic field to confirm the accuracy manually validation is more straightforward than with flow cytometry. With flow cytometry, micronucleated cells are normally not visualized, although it is possible to sort the target cells onto glass slides to confirm microscopically that the target cells are correct.

Flow cytometric analysis is rapid and provides the ability to analyze a large number of cells. A number of papers that demonstrate successful automated scoring methodologies have been published [4–19], although some of the earlier papers were focused on technological development rather than regulatory validation. Several publications have demonstrated that low dose effects of clastogens could be determined by analyzing large numbers of young erythrocytes that were not practical to evaluate by manual microscopy [20–25]. Additionally, recent interlaboratory data has shown that the reproducibility of flow cytometric analysis is much greater than microscopic scoring when a standard is used to calibrate the flow cytometry system [26,27]. Therefore it was the consensus of the group that flow cytometric analysis increases the capacity, and to a certain extent the sensitivity, of the *in vivo* micronucleus assay. As shown by Asano and colleagues [25], flow cytometry is capable of evaluating sufficient numbers of cells per animal such that variation among individuals becomes the limiting factor that defines the minimal micronucleus response that can be detected. Consequently whichever method is used, it is desirable that each laboratory should determine the minimum cell sample size required to ensure that scoring error is maintained below the level of animal-to-animal variation.

The group reconfirmed their view that data acquired by any automated method should be acceptable for regulatory use once it has met the validation criteria which were defined by the IWGT Micronucleus Working Group in Washington D.C., 1999 [3]. An important consensus agreement is that at the present time, sufficient validation has occurred for the flow cytometric CD71 method, as more extensive inter-laboratory collaborative data are available compared to the other procedures [24,26–28]. The outcomes of these inter-laboratory collaborative studies show good agreement between microscopy- and flow cytometry-generated data, as well as good intra- and inter-laboratory reproducibility for this methodology (see Appendices A and B).

During the discussions it was stated that data obtained by the flow cytometric analysis had already been evaluated and accepted by the regulatory authorities in the US [29] and EU (e.g., [30]).

### 1.2. Rat Blood reticulocytes for regulatory use

Accumulated evidence has established that it is acceptable to rely on the measurement of MN-reticulo-

cyte frequency in peripheral blood as well as bone marrow. This conclusion was restricted to the mouse in the ICH consensus because of presumed splenic elimination of MN-erythrocytes by the rat spleen [31,32]. In the second IWGT at Washington D.C. in 1999, we discussed the use of peripheral blood of rat as target cells to be analyzed as well as the use of mouse peripheral blood based on the Japanese collaborative trial data [33,34] (see Appendix C). However, the 1999 working group was unable to reach a consensus agreement because there was still some concern of rat spleen function. It was the consensus of the 2005 group that accumulated evidence shows that analysis of micronucleated peripheral blood reticulocytes is an acceptable cell population for the micronucleus assay in rats as well as mice [27]. Further, preliminary data suggest that peripheral blood may be an acceptable sampling tissue in other species including human [15,35–40]. In the case of humans as well as rats, the possible confounding effects of splenic activity will need to be considered (see Appendices A–C).

### *1.3. Micronucleus assays using tissues other than bone marrow*

One of the most important issues in the risk assessment of chemicals is the mode of action for carcinogenesis. At the present time it is considered that if the mechanism involves mutagenicity, then a threshold cannot be considered and a linear model would be used to set any potential acceptable daily intake (ADI). The Salmonella/microsome reverse mutation assay (as well as other *in vitro* assays) have played an important role in identifying whether a chemical is mutagenic or not. But, *in vitro* assay data cannot predict whether the chemical expresses its mutagenicity at the target site of carcinogenicity. Based on the weight of evidence philosophy, it is important to assess mutagenicity at the site of carcinogenesis. Theoretically transgenic animal models can assess gene mutations in any organ. On the other hand, chromosomal damage can be assessed only in proliferating organs and the bone marrow (hematopoietic) cells have been frequently used for this purpose. In the 2nd IWGT meeting in Washington D.C., we reviewed and evaluated the micronucleus assay using tissues other than bone marrow [3]. Here, we revisit the same topic again because the assessment of chromosomal damage using different organs has become more important and has been used increasingly for risk assessment purposes. We updated the tables of the micronucleus assay using tissues other than bone marrow including the collaborative validation trials organized by the Collaborative Study Group for the Micronucleus Test (CSGMT)/Japanese Environmental Mutagen Society (JEMS)/Mammalian Mutagenicity Study Group (MMS) [41–44] (see Appendix D). One of the major developments was the introduction of a new staining method using acridine orange together with DAPI that stains the cytoplasm and nucleus clearly and differentially [45].

A liver micronucleus assay with rats and mice was developed by Tates et al. [46,47], who used partial hepatectomy (PH) to stimulate liver cell proliferation. Subsequent investigators used mice [48–50], and induced liver cell proliferation by chemical damage, which induced mitogenesis, e.g., 4-acetylaminofluorene [51] or carbon tetrachloride [52], or used modifications of timing of treatments or sampling intervals [50,53]. The basic assay procedures and protocols have been established and validated [49], and approximately 40 chemicals have been tested so far. Several carcinogens that are negative or weakly positive in the erythrocyte micronucleus assay showed genotoxic effects in regenerating liver.

Recently, a hepatocyte micronucleus assay has been described in which liver of young rats is used without partial hepatectomy or chemical mitogen treatment [54–56]. The CSGMT/MMS engaged collaborative trials on this topic and the first report has been published [41]. The method does not need any additional treatment to detect MN induction by a test chemical in hepatocytes. The group agreed that the use of young rats as a model for detecting MN induction in the liver offers an alternative methodology to the use of partial hepatectomy or mitogenic stimulation based on the following analysis of published literature.

The metabolic capabilities of young rats have been studied and there was no essential difference in the total amount of cytochrome P450 between immature (50–60 g body weight) and adult (150–190 g body weight) in Long Evans rat [57–59]. It is well known that there is no difference among rat strains in main P450 species. In more detail, no difference between immature and adult in CYP2B1/2, immature > adult in CYP2A1 and CYP1A1 were reported [57]. Cytochromes P450 2B1/2 and 1A2 were determined in the growing neonate and fetus of control and 3-methylcholanthrene-pretreated rats. CYP1A2 activity was highest in the 1–2-week-old animals and then decreased with age. The inducibility of this activity by 3-methylcholanthrene was low in the young animals, but increased with age. CYP 2E1 in SD rat showed almost equivalent activity in 4-week-old animals compared to adult animals [60]. CYP2C11, which is the dominant P450 in SD male rat liver, of 4-week-old SD rat showed less activity than adult [58]. Gonzalez et al. [61]

reported that the activity of testosterone 6β-hydroxylase in 4-week-old male rat (strain unknown) was approximately a half of that of adult, but the content was high in the young rat and the total activity thought to be sufficient for the assay [61]. In summary, 4-week-old-rat liver can metabolize important xenobiotic chemicals, e.g., heterocyclic hydrocarbons, heterocyclic amines, nitrosamines, almost as effectively as in adult rats.

Additional experiences with colon and skin MN models has increased the databases enhancing confidence in the utility of these models.

Although not discussed at the meeting, recently germ-cell mutagenicity has come into the spotlight, e.g., WHO initiative project "Globally Harmonized System of Classification and Labeling of Chemicals (GHS)". At present, there are still only limited methods of determining germ cell mutagenesis *in vivo* (e.g., the specific locus test, dominant lethal test, transgenic mutation assays), and these are not frequently performed for regulatory use. The micronucleus assay using spermatids is an additional model that may be valuable for assessing chemical-induced germ cell mutation. The use of a micronucleus assay in mammalian germ cells was proposed in the early 1980s when two different approaches were described by Lähdetie and Parvinen [62] and by Tates et al. [63].

We reviewed and summarized assay results during the 2nd IWGT, Washington, 1999 [3]. We searched references to update the summary table but not many articles have been published after the last survey (Appendix D). One of the possible reasons might be that identification of aneugenesis detected by the FISH method or the use of simpler methods that determine DNA damage may be being used more frequently than cytogenetic analysis or measurement of mutations *per se*. Although we did not prepare a summary table, for readers' information we cite the references [64–70].

The table for the micronucleus assay results using fetal/neonatal animals by transplacental treatment was revised and is presented in Appendix D.

### *1.4. Relevance of a single-dose-level assay*

Under the current guidelines, e.g., OECD TG474 [71], the single-dose-level micronucleus assay is acceptable under limited conditions. The consensus during the IWGT meeting at Melbourne in 1993 was that negative data was acceptable from a study using a single dose level only when animals were treated at the limit dose of 2000 mg/kg and there was no depression of bone marrow proliferation and no sign of any adverse effects clinically. The single dose limit test is thus currently used just to demonstrate a clear negative response with non-toxic agents. As a basic guidance, for agents that are toxic below the 2000 mg/kg dose limit at least three dose levels separated by a factor between 2 and SQR (10) should be used [72].

The major concern with regard to the use of the single dose-level approach is the possibility of a 'downturn phenomenon'. Theoretically, the 'downturn phenomenon' should not occur when immature erythrocytes were targeted for analysis and the monotonic dose–response may be obtained when the sample time was optimized for each dose-level. The 'downturn phenomenon', however, has been shown to occur on some occasions although it is not clear whether this is due to excessive cytotoxicity within the bone marrow or to some other, as yet undefined, mechanism. In these cases, the group agreed, positive effects could still be obtained at the second highest dose. The use of a single dose level could sometimes lead to problems in data interpretation or to the loss of animals due to unexpected toxicity. In such cases it would be necessary to repeat the study with additional doses and this would lead to the use of an increased number of animals. After discussion of these issues, there was no consensus for the wider use of a single dose level protocol. Although it was agreed that it is important to reduce the number of experimental animals whenever possible, very few participants were in favor of such an approach in this case. There was an equal division among the participants concerning the need to use two or three doses-levels in this instance.

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## Appendix A

Inter-laboratory variation and reproducibility of micronucleus induction using model chemicals (cyclophosphamide, cisplatin, and vinblastine) evaluated in peripheral blood by flow cytometry, by methanol-fixed acridine orange staining, and acridine orange supravital staining, and also methanol-fixed acridine orange staining in bone marrow cells are summarized from an extensive validation study [27].

| Treatment | Intact/Splx | n | Lab | Avg. %MN-RET ± S.E.M. (fold increase) | | | |
|---|---|---|---|---|---|---|---|
| | | | | BM-AO | PB-AO | PB-SVAO | PB-FCM |
| 0 CP | Intact | 5 | L2 | | | | 0.13 ± 0.02 |
| | | 5 | L3 | | | | 0.16 ± 0.01 |
| | | 5 | L1 | 0.09 ± 0.03 | 0.15 ± 0.03 | | 0.12 ± 0.01 |
| | | 5 | L9 | 0.04 ± 0.01 | 0.10 ± 0.04 | | |
| | | 5 | L10 | 0.09 ± 0.04 | 0.05 ± 0.03 | | |
| | | 5 | L11 | 0.22 ± 0.08 | 0.18 ± 0.06 | | |
| | | 5 | L5 | | | 0.21 ± 0.07 | |
| | | 5 | L6 | | | 0.19 ± 0.04 | |
| | | 5 | L7 | | | 0.18 ± 0.02 | |
| 10 CP | Intact | 5 | L2 | | | | 1.39 ± 0.24 (10.7) |
| | | 5 | L3 | | | | 1.39 ± 0.22 (8.7) |
| | | 5 | L1 | 3.42 ± 0.28 (38.0) | 1.62 ± 0.33 (10.8) | | 1.15 ± 0.15 (9.6) |
| | | 5 | L9 | 1.52 ± 0.12 (38.0) | 0.88 ± 0.25 (8.8) | | |
| | | 5 | L10 | 1.92 ± 0.36 (21.3) | 0.41 ± 0.12 (8.2) | | |
| | | 5 | L11 | 2.74 ± 0.40 (12.5) | 1.72 ± 0.37 (9.6) | | |
| | | 5 | L5 | | | 2.11 ± 0.38 (10.0) | |
| | | 5 | L6 | | | 2.33 ± 0.31 (12.3) | |
| | | 5 | L7 | | | 1.23 ± 0.34 (6.8) | |
| 0 CP | Splx | 5 | L2 | | | | 0.17 ± 0.02 |
| | | 5 | L3 | | | | 0.19 ± 0.02 |
| | | 5 | L1 | 0.15 ± 0.02 | 0.16 ± 0.03 | | 0.15 ± 0.02 |
| | | 5 | L9 | 0.12 ± 0.03 | 0.13 ± 0.03 | | |
| | | 5 | L10 | 0.20 ± 0.04 | 0.09 ± 0.03 | | |
| | | 5 | L11 | 0.32 ± 0.08 | 0.14 ± 0.04 | | |
| | | 5 | L5 | | | 0.39 ± 0.07 | |
| | | 5 | L6 | | | 0.22 ± 0.03 | |
| | | 5 | L7 | | | 0.31 ± 0.05 | |
| 10 CP | Splx | 5 | L2 | | | | 2.19 ± 0.26 (12.9) |
| | | 5 | L3 | | | | 2.43 ± 0.25 (12.8) |
| | | 5 | L1 | 3.34 ± 0.29 (22.3) | 3.31 ± 0.40 (20.7) | | 2.03 ± 0.18 (13.5) |
| | | 5 | L9 | 1.39 ± 0.15 (11.6) | 1.37 ± 0.17 (10.5) | | |
| | | 5 | L10 | 1.95 ± 0.33 (9.8) | 0.61 ± 0.13 (6.8) | | |
| | | 5 | L11 | 3.06 ± 0.60 (9.6) | 2.56 ± 0.42 (18.3) | | |
| | | 5 | L5 | | | 3.74 ± 0.50 (9.6) | |
| | | 5 | L6 | | | 3.35 ± 0.24 (15.2) | |
| | | 5 | L7 | | | 1.84 ± 0.37 (5.9) | |
| CP Aggregate Correlation With Ref. Lab ($R^2$) | | 20 | L2 | | | | NA |
| | | 20 | L3 | | | | 0.9797 |
| | | 20 | L1 | NA | NA | | 0.9742 |
| | | 20 | L9 | 0.9273 | 0.8842 | | |
| | | 20 | L10 | 0.8551 | 0.8834 | | |
| | | 20 | L11 | 0.8512 | 0.9396 | | |
| | | 20 | L5 | | | NA | |
| | | 20 | L6 | | | 0.8901 | |
| | | 20 | L7 | | | 0.8173 | |
| 0 CisPl | Intact | 5 | L2 | | | | 0.08 ± 0.02 |
| | | 5 | L3 | | | | 0.09 ± 0.01 |
| | | 5 | L1 | 0.13 ± 0.03 | 0.08 ± 0.01 | | 0.09 ± 0.01 |
| | | 5 | L9 | 0.08 ± 0.03 | 0.09 ± 0.02 | | |
| | | 5 | L10 | 0.07 ± 0.04 | 0.05 ± 0.04 | | |
| | | 5 | L11 | 0.17 ± 0.03 | 0.20 ± 0.02 | | |
| | | 5 | L5 | | | 0.17 ± 0.08 | |
| 1.0 CisPl | Intact | 5 | L2 | | | | 0.53 ± 0.14 (6.6) |
| | | 5 | L3 | | | | 0.48 ± 0.12 (5.3) |
| | | 5 | L1 | 1.11 ± 0.24 (8.5) | 0.65 ± 0.14 (8.1) | | 0.48 ± 0.09 (5.3) |
| | | 5 | L9 | 0.32 ± 0.08 (4.0) | 0.41 ± 0.12 (4.6) | | |
| | | 5 | L10 | 0.33 ± 0.10 (4.7) | 0.28 ± 0.11 (5.6) | | |
| | | 5 | L11 | 0.62 ± 0.17 (3.6) | 0.88 ± 0.38 (4.4) | | |
| | | 5 | L5 | | | 0.85 ± 0.26 (5.0) | |
| 0 CisPl | Splx | 5 | L2 | | | | 0.16 ± 0.02 |
| | | 5 | L3 | | | | 0.16 ± 0.02 |
| | | 5 | L1 | 0.14 ± 0.03 | 0.17 ± 0.03 | | 0.15 ± 0.02 |

| Treatment | Intact/Splx | n | Lab | Avg. %MN-RET ± S.E.M. (fold increase) | | | |
|---|---|---|---|---|---|---|---|
| | | | | BM-AO | PB-AO | PB-SVAO | PB-FCM |
| | | 5 | L9 | 0.11 ± 0.01 | 0.12 ± 0.01 | | |
| | | 5 | L10 | 0.06 ± 0.02 | 0.22 ± 0.04 | | |
| | | 5 | L11 | 0.17 ± 0.07 | 0.29 ± 0.07 | | |
| | | 5 | L5 | | | 0.15 ± 0.02 | |
| 1.0 CisPl | Splx | 5 | L2 | | | | 0.89 ± 0.18 (5.6) |
| | | 5 | L3 | | | | 0.91 ± 0.19 (5.7) |
| | | 5 | L1 | 1.06 ± 0.25 (7.6) | 1.12 ± 0.17 (6.6) | | 0.90 ± 0.14 (6.0) |
| | | 5 | L9 | 0.38 ± 0.13 (3.5) | 0.54 ± 0.11 (4.5) | | |
| | | 5 | L10 | 0.52 ± 0.12 (8.7) | 1.12 ± 0.20 (5.1) | | |
| | | 5 | L11 | 1.05 ± 0.30 (6.2) | 0.86 ± 0.05 (3.0) | | |
| | | 5 | L5 | | | 1.11 ± 0.23 (7.4) | |
| CisPl Aggregate | | 20 | L2 | | | | NA |
| Correlation | | 20 | L3 | | | | 0.9846 |
| With Ref. Lab | | 20 | L1 | NA | NA | | 0.9555 |
| ($R^2$) | | 20 | L9 | 0.5165 | 0.8336 | | |
| | | 20 | L10 | 0.7554 | 0.7481 | | |
| | | 20 | L11 | 0.769 | 0.5104 | | |
| 0 VB | Intact | 5 | L2 | | | | 0.10 ± 0.01 |
| | | 5 | L3 | | | | 0.11 ± 0.02 |
| | | 5 | L1 | 0.10 ± 0.02 | 0.13 ± 0.03 | | 0.16 ± 0.01 |
| | | 5 | L9 | 0.06 ± 0.02 | 0.07 ± 0.02 | | |
| | | 5 | L10 | 0.18 ± 0.05 | 0.08 ± 0.01 | | |
| | | 5 | L11 | 0.15 ± 0.03 | 0.24 ± 0.03 | | |
| | | 3 | L5 | | | 0.34 ± 0.07 | |
| 0.125 VB | Intact | 3 | L2 | | | | 0.43 ± 0.08 (4.3) |
| | | 3 | L3 | | | | 0.41 ± 0.05 (3.7) |
| | | 5,3,3 | L1 | 1.46 ± 0.18 (14.6) | 0.57 ± 0.09 (4.4) | | 0.47 ± 0.07 (2.9) |
| | | 5,3 | L9 | 0.65 ± 0.13 (10.8) | 0.43 ± 0.15 (6.1) | | |
| | | 5,5 | L10 | 0.51 ± 0.11 (2.8) | 0.11 ± 0.05 (1.4) | | |
| | | 5,3 | L11 | 0.70 ± 0.13 (4.7) | 0.32 ± 0.06 (1.3) | | |
| | | 2 | L5 | | | 0.65 ± 0.20 (1.9) | |
| 0 VB | Splx | 4 | L2 | | | | 0.17 ± 0.03 |
| | | 4 | L3 | | | | 0.20 ± 0.02 |
| | | 4 | L1 | 0.12 ± 0.01 | 0.21 ± 0.03 | | 0.20 ± 0.02 |
| | | 4 | L9 | 0.04 ± 0.01 | 0.04 ± 0.02 | | |
| | | 4 | L10 | 0.11 ± 0.03 | 0.09 ± 0.02 | | |
| | | 4 | L11 | 0.26 ± 0.08 | 0.30 ± 0.12 | | |
| | | 4 | L5 | | | 0.35 ± 0.10 | |
| 0.125 VB | Splx | 5 | L2 | | | | 0.57 ± 0.19 (3.4) |
| | | 5 | L3 | | | | 0.72 ± 0.18 (3.6) |
| | | 5,3,5 | L1 | 0.87 ± 0.24 (7.3) | 0.60 ± 0.20 (2.9) | | 0.78 ± 0.22 (3.9) |
| | | 5 | L9 | 0.34 ± 0.16 (8.5) | 0.23 ± 0.10 (5.8) | | |
| | | 5,5 | L10 | 0.51 ± 0.15 (4.6) | 0.13 ± 0.03 (1.4) | | |
| | | 5 | L11 | 0.75 ± 0.25 (2.9) | 0.36 ± 0.08 (1.2) | | |
| | | 3 | L5 | | | 0.95 ± 0.53 (2.7) | |
| VB Aggregate | | 17 | L2 | | | | NA |
| Correlation | | 17 | L3 | | | | 0.9321 |
| With Ref. Lab | | 17 | L1 | NA | NA | | 0.9584 |
| ($R^2$) | | 19, 15 | L9 | 0.8134 | 0.3666 | | |
| | | 19, 15 | L10 | 0.5644 | 0.0278 | | |
| | | 19, 15 | L11 | 0.5436 | 0.0992 | | |

Abbreviations: MN-RET, micronucleated reticulocyte; Splx, splenectomized; BM-AO, bone marrow scored by microscopy using standard acridine orange staining of methanol-fixed slides; PB-AO, peripheral blood scored by microscopy using standard AO staining of methanol-fixed slides; PB-SVAO, peripheral blood scored by microscopy using supravital acridine orange staining; PB-FCM, peripheral blood scored by flow cytometry using the propidium iodide/anti-CD71-based labeling method; S.E.M., standard error of the mean; *by "aggregate correlation" it is meant that the MN-RET value obtained for every animal in a particular study obtained at a reference laboratory (L1, L2 or L5) was plotted against the corresponding value obtained at each other laboratory. The $R^2$ values shown here were calculated in Microsoft Excel. Experimental design: Groups of intact and splenectomized Sprague Dawley male rats were administered vehicle, cyclophosphamide (10 mg/kg/day), cisplatin (1 mg/kg/day) or vinblastine (0.125 mg/kg/day) for 5 days at 24 h intervals (five animals per group). Twenty-four hours after the last administration, blood and bone marrow were prepared as coded slides or fixed blood suspensions and supplied to several laboratories that have extensive experience performing micronucleus measurements. MN-RET averages, S.E.M., fold-increases, and correspondence to reference laboratories are presented. In some instances, the number of measurements ($n$) performed per chemical does not equal 20. Except for Vinblastine Splx Control rats where $n = 4$, instances of $n < 5$ were due to excessive toxicity. More detailed information regarding these experiments can be found in MacGregor et al. [27].

## Appendix B

Variation and reproducibility of intra- and inter-laboratory scoring of blinded triplicate samples are summarized from an extensive validation study [26].

| Laboratory | Method | Compartment | Treatment | %MN-RET | | |
|---|---|---|---|---|---|---|
| | | | | Avg. ± S.E.M. | %CV | Fold difference |
| L1 | MeOH-AO | BM | Vehicle | 0.15 ± 0.03 | 33.3 | |
| | | | CP | 3.35 ± 0.10 | 5.4 | 22.3 |
| L9 | MeOH-AO | BM | Vehicle | 0.05 ± 0.05 | 173.2 | |
| | | | CP | 1.63 ± 0.27 | 28.4 | 32.6 |
| L10 | MeOH-AO | BM | Vehicle | 0.03 ± 0.02 | 86.6 | |
| | | | CP | 2.33 ± 0.23 | 17.3 | 77.7 |
| L11 | MeOH-AO | BM | Vehicle | 0.18 ± 0.03 | 31.5 | |
| | | | CP | 2.44 ± 0.20 | 13.9 | 13.6 |
| *Pooled* L1, 9, 10, 11* | | | *Vehicle* | *0.10 ± 0.02* | *80.5* | |
| | | | *CP* | *2.44 ± 0.21* | *29.1* | *24.4* |
| L1 | MeOH-AO | PB | Vehicle | 0.05 ± 0.03 | 100.0 | |
| | | | CP | 1.77 ± 0.17 | 16.6 | 35.4 |
| L9 | MeOH-AO | PB | Vehicle | 0.05 ± 0.00 | 0.0 | |
| | | | CP | 0.50 ± 0.03 | 10.0 | 10.0 |
| L11 | MeOH-AO | PB | Vehicle | 0.18 ± 0.04 | 41.7 | |
| | | | CP | 1.42 ± 0.10 | 12.3 | 7.9 |
| *Pooled L1, 9, 11* | | | *Vehicle* | *0.09 ± 0.03* | *85.6* | |
| | | | *CP* | *1.23 ± 0.20* | *48.2* | *13.7* |
| L5 | SV-AO | PB | Vehicle | 0.13 ± 0.03 | 43.3 | |
| | | | CP | 1.83 ± 0.15 | 13.7 | 14.1 |
| L6 | SV-AO | PB | Vehicle | 0.12 ± 0.07 | 99.0 | |
| | | | CP | 1.77 ± 0.32 | 31.2 | 14.8 |
| L7 | SV-AO | PB | Vehicle | 0.22 ± 0.14 | 113.8 | |
| | | | CP | 1.47 ± 0.27 | 31.7 | 6.7 |
| *Pooled L5, 6, 7* | | | *Vehicle* | *0.16 ± 0.05* | *94.3* | |
| | | | *CP* | *1.69 ± 0.14* | *24.7* | *10.6* |
| L1 | FCM | PB | Vehicle | 0.12 ± 0.02 | 24.8 | |
| | | | CP | 0.99 ± 0.04 | 6.5 | 8.3 |
| L2 | FCM | PB | Vehicle | 0.11 ± 0.02 | 31.5 | |
| | | | CP | 1.04 ± 0.04 | 6.7 | 9.5 |
| L3 | FCM | PB | Vehicle | 0.11 ± 0.02 | 32.9 | |
| | | | CP | 1.11 ± 0.04 | 6.8 | 10.1 |
| *Pooled L1, 2, 3* | | | *Vehicle* | *0.11 ± 0.01* | *26.5* | |
| | | | *CP* | *1.05 ± 0.03* | *7.6* | *9.5* |

Abbreviations: MN-RET, micronucleated reticulocyte; MeOH-AO, acridine orange staining of methanol-fixed smears; SV-AO, supravital staining using acridine orange-coated slides; FCM, flow cytometry; BM, bone marrow; PB, peripheral blood; CP, cyclophosphamide; S.E.M., standard error of the mean; %CV, percent coefficient of variance.

Experimental design: One female Sprague Dawley rat was administered vehicle or cyclophosphamide (10 mg/kg/day) for 6 days at 24 h intervals. Twenty-four hours after the last administration, blood and bone marrow were prepared as coded slides or fixed blood suspensions and supplied to several laboratories that have extensive experience performing micronucleus measurements. The vehicle and the cyclophosphamide specimens were each supplied as three uniquely coded slides or tubes for analysis. MN-RET averages, S.E.M., and mean fold increase for each laboratory are presented. *Like-method data from three or four labs were combined for "pooled" calculations. More detailed information regarding these experiments can be found in Dertinger et al. [26].

## Appendix C

Accumulated evidence has established that it is acceptable to rely on the measurement of MN-reticulocyte frequency in peripheral blood as well as bone marrow. In the second IWGT at Washington D.C. in 1999, we have discussed the use of peripheral blood of rat as target cells to be analyzed as well as mouse based on the Japanese collaborative trial data [33,34]. As an appendix, we summarize here again the outcomes of Japanese collaborative trial to evaluate the relevancy of using rat peripheral blood reticulocytes as target cells to be analyzed. The negative control values were $0.14 \pm 0.12\%$ in bone marrow (252 rats) and $0.07 \pm 0.08\%$ in peripheral blood (287 rats). The positive control (cyclophosphamide, 20 mg/kg, oral gavage, sampled 24 h after the last treatment) were $1.8 \pm 0.8\%$ in bone marrow (56 rats) and $0.80 \pm 0.48\%$ in peripheral blood (77 rats). The Table shows the summary table of the collaborative trial on 16 model chemicals that induce micronuclei with different modes of action. Although the absolute values of micronucleated reticulocyte frequencies were different between observations of bone marrow cells and peripheral reticulocytes in negative and positive controls and also model chemicals, the statistically significant induction of micronucleated reticulocytes were observed on the positive control chemical (cyclophosphamide) and tested model chemicals.

| Chemicals | Species | Route | Bone marrow MNPCE Dose (mg/kg) | (%) | Fold Inc. | Peripheral blood MNPCE Dose (mg/kg) | (%) | Fold Inc. |
|---|---|---|---|---|---|---|---|---|
| *p*-Aminoazobenzene | Rat | ip | 80 | 0.41 | 3.40 | 80 | 0.38 | 3.50 |
| | Mouse | ip | 150 | 0.70 | 5.00 | 150 | 1.14 | 8.10 |
| *o*-Aminoazotoluene | Rat | ip | 600 | 0.11 | 0.90 | 600 | 0.16 | 8.00 |
| | Mouse | ip | 400 | 0.60 | 3.00 | | | |
| Benzene | Rat | po | 2000 | 2.34 | 16.7 | 2000 | 2.16 | 18.0 |
| | Mouse | po | 2000 | 2.75 | 34.4 | 2000 | 2.75 | 6.10 |
| Diepoxybutane | Rat | po | 144 | 4.00 | 22.2 | 144 | 0.78 | 26.0 |
| | Mouse | po | 72 | 8.68 | 36.2 | | | |
| Ethyl methanesulfonate | Rat | ip | 300 | 1.36 | 45.3 | 300 | 0.75 | 12.5 |
| | Mouse | ip | 200 | 1.48 | 8.20 | | | |
| *N*-Ethyl-*N*-nitrosourea | Rat | ip | 75 | 2.78 | 27.8 | 75 | 1.36 | 12.4 |
| | Mouse | ip | 100 | 7.85 | 60.4 | 50 | 3.78 | 11.8 |
| 5-Fluorouracil | Rat | ip | 40 | 1.39 | 10.7 | 80 | 0.69 | 34.5 |
| | Mouse | ip | 50 | 3.13 | 24.1 | 50 | 2.54 | 18.1 |
| Hydrazine | Rat | ip | 75 | 0.58 | 2.80 | 12.5 | 0.15 | 2.50 |
| | Mouse | ip | | | | 50 | 1.18 | 5.90 |
| 6-Mercaptopurine | Rat | ip | 50 | 0.92 | 6.10 | 25 | 0.53 | 5.30 |
| | Mouse | ip | 50 | 6.98 | 27.9 | 25 | 2.9 | 7.30 |
| MNNG | Rat | ip | 100 | 1.20 | 4.00 | 100 | 0.75 | 3.75 |
| | Mouse | ip | 150 | 2.55 | 10.2 | 100 | 0.8 | 3.10 |
| Nitrogen mustard | Rat | ip | 2 | 3.24 | 23.5 | 2 | 0.66 | 7.50 |
| | Mouse | ip | 2 | 7.17 | 29.6 | 2 | 4.98 | 21.4 |
| 1,3-Propane sultone | Rat | ip | 60 | 2.15 | 13.4 | 30 | 0.66 | 7.30 |
| | Mouse | ip | | | | 72 | 1.92 | 11.3 |
| Propylene oxide | Rat | ip | 200 | 0.33 | 2.20 | 300 | 0.4 | 3.1 |
| | Mouse | ip | 300 | 0.53 | 8.80 | 300 | 0.4 | 6.7 |
| Triethylenemelamine | Rat | ip | 0.5 | 5.58 | 11.8 | 0.13 | 1.08 | 6.80 |
| | Mouse | ip | 1 | 7.52 | 32.7 | 1 | 6.64 | 66.4 |
| Urethane | Rat | ip | 1000 | 1.29 | 11.7 | 1000 | 1.6 | 40.0 |
| | Mouse | ip | | | | 1000 | 3.28 | 16.4 |
| Vincristine | Rat | ip | 0.2 | 0.99 | 7.60 | 0.08 | 0.26 | 4.3 |
| | Mouse | ip | 0.125 | 11.68 | 89.8 | 0.3 | 4.34 | 54.3 |

Light shadow: frequency in mice was more than twice that in rats. Dark shadow: frequency in rats was more than twice that in mice. *MS/Ae strain mice.

## Appendix D

Micronucleus assay results using tissues other than haematopoietic tissue have become increasingly important for risk assessment to assess chemical clastogenicity at the target site of carcinogenesis or other adverse effects. We have summarized the data on micronucleus induction in liver, colon, and skin in Table D.1 including new data published after the 2nd IWGTP report. In Table D.2, we add new data on the micronucleus assay results on germ cells (mainly spermatids). These data using germ cells are more weighted in "Globally Harmonized System of Classification and Labeling of Chemicals (GHS)" for labeling of chemicals. Table D.3 shows micronucleus assay results using fetal/neonatal animals by transplacental treatment.

Table D.1
Micronucleus assay results using liver, colon, and skin

| [Specific tissue/organ] Chemical | MN in erythrocyte | | MN in the specific tissue/organ | |
|---|---|---|---|---|
| | Result | Reference | Result | Reference |
| [Liver] | | | | |
| 2-Acetylaminofluorene | + | [76] | +/− | [50,51,54,77] |
| 4-Acetylaminofluorene | − | [78] | − | [51] |
| Acrylamide | I | [79] | + | [49] |
| 4-Aminobiphenyl | + | [79] | + | [48] |
| Amsacrine (*m*-AMSA) | + | [50] | + | [50] |
| *o*-Anthranilic acid | − | [78] | − | [77] |
| L-Ascorbic acid | − | [81] | − | [77] |
| Auramine O | − | [79] | + | [49] |
| Benzene | + | [76] | − | [50,80] |
| e-Caprolactam | − | [81] | − | [77] |
| Carbon tetrachloride | − | [79] | − | [80] |
| Clofibrate | | | + | [49] |
| 4-Chloro-*o*-phenylenediamine (CPDA) | + | [33] | − | [41] |
| Cyclophosphamide | + | [76] | +/− | [50,54,77] |
| 2,4-Diaminotoluene | I | [79] | + | [77] |
| Di(2-ethylhexyl)phthalate (DEHP) | − | [79] | − | [41] |
| Diethylnitrosamine | − | [79] | + | [46–48,50,54,56] |
| *p*-Dimethylamino-azobenzene (DAB) | − | [79] | + | [41] |
| 4-Dimethylamino-3′-methylazobenzene | − | [50] | + | [50] |
| 6-Dimethylaminophenylazo-benzthiazole | − | | + | [51] |
| 7,12-Dimethylbenz[*a*]anthracene | + | [76] | − | [54] |
| Dimethylnitrosamine | + | [76] | + | [41,46–49,51] |
| | −/+ | [73,74] | | |
| 1,1-Dimethylhydrazine | + | [79] | + | [48,77] |
| 1,2-Dimethylhydrazine | + | [79] | + | [51] |
| 2,4-Dinitrotoluene | − | [75] | + | [77] |
| 1,4-Dioxane | I/− | [79,82] | + | [82] |
| Ethylmethanesulfonate | + | [76] | − | [53] |
| Ethylnitrosourea | + | [76] | + | [47,50] |
| 5-Fluorouracil | + | [76] | + | [50] |
| Kojic acid | + | [41] | − | [41] |
| D-Mannitol | − | [81] | − | [77] |
| Methoxychlor | − | [77] | − | [77] |
| 4,4′-Methylenedianiline (MDA) | − | [41] | − | [41] |
| Methylmethanesulfonate | + | [76] | − | [41,47] |
| Mitomycin C | + | [76] | + | [50,54,77] |
| 2-Nitrofluorene | − | [54] | + | [54] |
| *N*-Nitrosomorpholine | + | [79] | + | [83] |
| Phenazopyridine hydrochloride | + | [79] | + | [77] |
| Potassium chromate (IV) ($K_2CrO_4$) | + | [76] | + | [50] |
| β-Propiolactone | − | [79] | + | [48] |
| 4-*N*-Pyrrolindinylazobenzene | | | − | [51] |
| Quinoline | I | [41] | + | [41] |
| Styrene oxide | − | [79] | + | [49] |

Table D.1 (*Continued*)

| [Specific tissue/organ] Chemical | MN in erythrocyte | | MN in the specific tissue/organ | |
|---|---|---|---|---|
| | Result | Reference | Result | Reference |
| Selenious acid ($H_2SeO_3$) | + | [50] | + | [50] |
| *o*-Toluidine | + | [41] | − | [41] |
| [Colon epithelium] | | | | |
| Carbendazim | + | [84–86] | + | [87] |
| | − | [87] | | |
| Colchicine | + | [76,87–91] | + | [87] |
| Cyclophosphamide | + | [76,89,92,93] | + | [93] |
| 1,2-Dimethylhydrazine | + | [79] | + | [42,87,93–96] |
| | − | [87,92,93,96] | | |
| Griseofulvin | − | [79,87] | + | [87] |
| Methylnitrosourea | + | [79] | + | [42] |
| Mitomycin C | + | [76,89] | + | [42] |
| Tubulazole | + | [87,89,91] | + | [87] |
| [Skin] | | | | |
| Acetone | − | [97] | −[a] | [98,99] |
| | | | − | [43] |
| 2-acetylaminofluorene (2-AAF) | + | [100] | + | [101] |
| Anthracene | | | − | [102] |
| Benz[*a*]anthracene | I | [79] | + | [102] |
| Benzene (BEN) | + | [76] | − | [101] |
| Benzo[*a*]pyrene | + | [76] | +[a] | [44,98,102,103] |
| Benzo[*e*]pyrene | | | − | [102] |
| Catechol | + | [109] | − | [101] |
| Chrysene | | | +[a]− | [98,102] |
| Clinafloxacin(CLFX) + UVA | | | + (oral) | [104] |
| Colchicine (COL) | + | [76] | + | [101] |
| Cyclophosphamide | + | [76] | − | [101] |
| | | | + | [105] |
| Dibenz[*a,c*]anthracene | | | + | [102] |
| Dibenz[*a,h*]anthracene | + | [79] | + | [102] |
| Dichlorvos | − | [79] | +[a] | [106] |
| Diethylstilbesterol (DES) | − | [76] | − | [101] |
| 15,16-Dihydrocyclopenta[*a*]-phenanthrene-17-one | | | − | [103] |
| 15,16-Dihydro-11-methylcyclo-penta[*a*]phenanthrene-17-one | | | + | [103] |
| 7,12-Dimethylbenz[*a*]anthracene | + | [76] | +[a] | [98,99,107] |
| | | | + | [44,102] |
| 1,2-Dimethyl hydradine dihydrocloride | + | [79] | + | [101] |
| 17-β-Estradiol | − | [79] | − | [101] |
| 2-Ethyl-1,3-hexanediol | | | − | [105] |
| *N*-Ethyl-*N′*-nitro-*N*-nitrosoguanidine (ENNG) | + | [108] | + | [44,105] |
| 5-Fluorouracil (5-FU) | + | [76] | + | [101] |
| Hydrazine HCl (HDZ) | + | [79] | − | [101] |
| Hydroquinone | + | [109] | − | [101] |
| *o*-Hydroxybiphenyl | | | − | [101] |
| Levofloxacin (LVFX) + UVA | | | − (oral) | [104] |
| Lomefloxacin (LFLX) + UVA | | | + (oral) | [104] |
| 6-Mercaptopurine | + | [76] | + | [101] |
| 3-Methylcholanthrene | | | + | [102] |
| Methyl methanesulfonate | + | [76] | +[a] | [99] |
| | | | + | [43,101] |
| *N*-Methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) | + | [76] | + | [44] |
| Mitomycin C | + | [76] | + | [43,105] |

Table D.1 (*Continued*)

| [Specific tissue/organ] Chemical | MN in erythrocyte | | MN in the specific tissue/organ | |
|---|---|---|---|---|
| | Result | Reference | Result | Reference |
| Nickel chloride ($NiCl_2$) | – | [79] | I | [101] |
| *p*-Nitrophenol | | | – | [105] |
| 4-Nitroquinoline 1-oxide (4NQO) | + | [110] | + | [44] |
| *N*-Nitrosodiethylamine (DEN) | – | [79] | – | [101] |
| | + (Liver) | [56] | | |
| Pyrene | | | – | [98,102] |
| TPA | | | – | [99] |
| TPA + mezerein | | | + | [111] |
| Trichloroethylene | | | – | [105] |
| Triethylenemelamine | + | [76] | + | [112] |
| Urethane | + | [76] | + | [98] |
| Vinblastine | + | [113] | + | [101,105] |

Abbreviations: TPA: Phorbol-12-myristate-13-acetate; +: positive, –: negative, I: inconclusive (i.e., there were contradictory results from different laboratories).

[a] In vivo/in vitro method.

Table D.2
Micronucleus assay results on spermatids

| Compound | Method[a] | Species tested[b] | No. of doses | No. of animals | No. of SPD/animal | Stages tested | Results | Reference |
|---|---|---|---|---|---|---|---|---|
| Acrylamide | S | M | 3 | 4–5 | 1000–2000 | Diakinesis-MI/MII | – | [114] |
| | | | | | | Diff. SPG-preleptotene | + | |
| Acrylamide | D | R | 3 | 5 | 2000 | Diplotene diakinesis | – | [115] |
| | | | | | | Late pachytene | – | |
| | | | | | | Preleptotene | + | |
| Acrylamide | S | R | 3 | 5 | 1000 | DIAKINESIS | – | [116] |
| | | | | | | Diplotene | – | |
| | | | | | | Diff. SPG/preleptotene | + | |
| Butadiene | S | M | 3 | 5 | 2000 | Late meiotic prophase | – | [117] |
| | | | | | | Diff. SPG/prelept./early prophase | + | |
| Butadiene | S | M | 3 | 3–5 | 1000 | Late meiotic prophase | – | [118] |
| | | | | | | Prelept./early prophase | – | |
| | | | | | | Diff. SPG/preleptotene | + | |
| Butadiene dioloepoxide | D | R | 2 | 4–5 | 2000 | Diplotene diakinesis | + | [119] |
| | | | | | | Late pachytene | + | |
| | | | | | | Preleptotene | + | |
| Carbendazim | D | R | 3 | 6 | 1500 | Stage I | + | [120] |
| | | | | | | Stage V | + | |
| | | | | | | Stage VII | – | |
| Chloral hydrate | S | M | 3 | 5 | 1000 | Diakinesis-MI | – | [121] |
| | | | | | | Leptotene-zygotene | – | |
| | | | | | | Preleptotene | + | |
| | | | | | | Stem cell SPG | + | |
| | S | M | 1 | 2–3 | 2000 | Pachytene-diakinesis | – | [122] |
| | | | | | | Preleptoten | – | |
| | | | | | | Stem cell SPG | + | |
| 1,2,3,4-Diepoxybutane | S | M | 2 | 5 | 2000 | Diakinesis/meiotic divisions | + | [123] |
| | | | | | | Diff. SPG/preleptotene | + | |
| 1,2,3,4-Diepoxybutane | S | M | 2 | 4–5 | 1000 | Diakinesis | – | [118] |
| | | | | | | Diplotene | + | |
| | | | | | | Zygotene | – | |
| | | | | | | Diff. SPG-preleptotene | – | |

Table D.2 (*Continued*)

| Compound | Method[a] | Species tested[b] | No. of doses | No. of animals | No. of SPD/animal | Stages tested | Results | Reference |
|---|---|---|---|---|---|---|---|---|
| 1,2,3,4-Diepoxybutane | D | R | 2 | 4–5 | 2000 | Diplotene diakinesis | + | [119] |
| | | | | | | Late pachytene | – | |
| | | | | | | Preleptotene | + | |
| | | | | | | Stem cell SPG | + | |
| 1,2,3,4-Diepoxybutane | S | R | 4 | 4–5 | 1000 | Diakinesis | – | [118] |
| | | | | | | Diplotene | + | |
| | | | | | | Zygotene | + | |
| | | | | | | Diff. SPG-preleptotene | + | |
| 3,4-Epoxybutene | S | M | 3 | 5 | 2000 | Diakinesis/MI/MII | – | [123] |
| | | | | | | Diff. SPG/preleptotene | + | |
| 3,4-Epoxybutene | S | M | 2 | 5 | 1000 | Diakinesis | – | [118] |
| | | | | | | Diplotene | – | |
| | | | | | | Diff. SPG-preleptotene | + | |
| 3,4-Epoxybutene | D | R | 5 | 4–5 | 2000 | Diplotene diakinesis | + | [119] |
| | | | | | | Late pachytene | + | |
| | | | | | | Preleptotene | + | |
| | | | | | | Stem cell SPG | – | |
| 3,4-Epoxybutene | S | R | 2 | 4–5 | 1000 | Diakinesis | + | [118] |
| | | | | | | Diplotene | + | |
| | | | | | | Diff. SPG-preleptotene | + | |
| Etoposide | D | R | 2 | 2–5 | 1000 | Diplotene diakinesis | + | [115] |
| | | | | | | Late pachytene | + | |
| | | | | | | Preleptotene | + | |
| | D | M | 1 | 3 | 1000 | Diplotene-diakinesis | + | [124] |
| Merbarone | D | M | 2 | 3 | 1000 | Diplotene-diakinesis (CREST) | + | [124] |
| | | | | | | Prometaphase-MI | – | |
| | | | | | | Prometaphase-MII | – | |
| Potassium bromate | S | M | 3 | 10 | 2000 | Spermatogonial stem cells–spermatogomia/ spermatocytes | – | [125] |
| Trichloroethylene | S | M | 3 | 6 | 1000 | Preleptotene-early pachytene | + | [121] |
| Trophosphamide | S | M | 2 | 4–5 | 1000 | Diakinesis/MI/MII | – | [126] |
| | | | | | | Diff. SPG-preleptotene | + | |
| Trophosphamide | D | R | 2 | 5 | 2000 | Dipl.diak. | + | [127] |
| | | | | | | Late pachytene | + | |
| | | | | | | Preleptotene | + | |
| Merbarone | D | M | 2 | 3 | 1000 | Diplotene-diakinesis | + | CREST |
| | | | | | | Prometaphase-MI | – | [124] |
| | | | | | | Prometaphase-MII | – | |
| VP-16 | D | M | 1 | 3 | 1000 | Diplotene-diakinesis | + | CREST [124] |
| Carbendazim | D | R | 3 | 6 | 1500 | Stage I | + | CREST |
| | | | | | | Stage V | + | [120] |
| | | | | | | Stage VII | – | |
| Potassium bromate | S | M | 3 | 10 | 2000 | Spermatogonial stem cells–spermatogomia/ spermatocytes | – | In drinking water for 8 weeks, antikinetochore [125] |
| Chloral hydrate | S | M | 1 | 2–3 | 2000 | Pachytene-diakinesis | – | CREST, FISH |
| | | | | | | Preleptoten | – | [128] |
| | | | | | | Stem cell SPG | + | |

[a] S: suspension method, D: dissection method.

[b] M: mouse, R: rat.

Table D.3
Micronucleus assay results using feta/neonatal animals by transplacental treatment

| Reference | Species/tissue | Test agent | Treatment regimen | Results/comments |
|---|---|---|---|---|
| [129] | Mouse; maternal bone marrow, fetal liver, and fetal blood | γ-Radiation, MMS, procarbazine, MMC, B[*a*]P | i.p. injection during 15–16th day of pregnancy | Increased MN in maternal bone marrow, fetal liver and fetal blood by all test agents |
| [130] | Mouse; maternal bone marrow, fetal liver, neonatal blood | DEN | i.p. injection during 15–16th day of pregnancy | Increased MN in fetal liver and neonatal blood; no increase in MN in maternal bone marrow |
| [131] | Mouse; maternal bone marrow, and fetal liver | γ-Radiation, MMS, procarbazine, MMC, B[*a*]P, cyclophosphamide, DEN | i.p. injection on 16th day of pregnancy | Increased MN in maternal bone marrow, and fetal liver by all test agents, except DEN which was positive only in fetal liver; fetal liver was relatively more sensitive |
| [132] | Mouse; maternal bone marrow, fetal liver, and fetal blood | Cyclophosphamide, triethylenemelamine, methotrexate | Various gestation days | Fetal liver MN test is useful to detect mutagens and embryotoxicity |
| [133] | Mouse; maternal bone marrow and fetal liver | MNNG, cyclophosphamide, methotrexate, 5-fluorouracil, 6-mercaptopurine, triethylene-melamine | Various routes on 13th day of pregnancy and/or 5 times daily | Increased MN in maternal bone marrow, and fetal liver by all test agents, except MNNG, which was positive only in fetal liver; fetal liver was relatively more sensitive |
| [134] | Rat; maternal bone marrow, embryonic blastocytes | Chlorambucil | i.p. injection on gestation days 1–3 | Increased MN in maternal bone marrow and embryonic blastocytes; the latter being more sensitive |
| [135] | Mouse; fetal liver | Cigarette smoke | Inhalation on 15th and 16th day of gestation, long-term exposure on premating through mating and/or 16th day of gestation | No increased MN in fetal liver cells; but a sister chromatid exchange assay was positive under these conditions |
| [136] | Mouse and rat; maternal bone marrow, fetal liver, fetal blood | MMC, DMBA | MMC: ip on 12th to 17th day for mouse, DMBA: oral on 11th to 16th day for mouse and 13th to 18th day for rat | Increased MN in all tissues studied, with fetal blood cells being more sensitive |
| [137] | Mouse; maternal bone marrow and fetal liver | B[*a*]P | Various gestation days | Increased MN in both tissues studied, with fetal liver cells being more sensitive |
| [138] | Mouse; maternal bone marrow and fetal liver | 4-Nitroquinoline 1-oxide | s.c. dosing on 12th, 14th, or 16th day of gestation | Increased MN in both tissues studied, with fetal liver cells being more sensitive based on equal adduct levels |
| [139] | Mouse; maternal bone marrow, fetal liver, and fetal blood | Benzene | i.p. injections on 14th day of gestation | Increased MN in all tissues studied, with fetal liver cells being more sensitive |
| [140] | Mouse; maternal bone marrow and fetal liver | Benzidine, heliotrine, monocrotaline, urethane | i.p. injection on 17th–19th days of gestation | Increased MN in both tissues studied, with fetal liver cells being more sensitive |
| [141] | Mouse; fetal blood | MMS, heliotrine, lasiocarpine, monocrotaline, B[*a*]P | i.p. or p.o. on day 15 or 16 of gestation | Increased MN in fetal blood erythrocytes, both young (uniformly-stained RNA) and older (stippled) RNA-positive erythrocytes |
| [142] | Mouse; maternal bone marrow, fetal liver, and fetal blood | Cyclophosphamide, DEN, *N*-nitroso-*N*-ethylurea, MMC | i.p. injections on 15th or 16th day of gestation | Increased MN in maternal bone marrow, fetal liver, and fetal blood by all test agents, except DEN, which was positive only in fetal liver and fetal blood |

Table D.3 (*Continued*)

| Reference | Species/tissue | Test agent | Treatment regimen | Results/comments |
|---|---|---|---|---|
| [79] | Mouse; maternal bone marrow, fetal blood | Diethylstilbestrol | i.p. injection on 15th–16th day of gestation | Increased MN in fetal blood, but only marginal in maternal bone marrow |
| [143] | Mouse; 4-day embryo (blastocysts or morulas) | Acrylamide | i.p. injection for 5 consecutive days. Untreated C3H/J female mice were mated with treated C57BL/6J mice 5–17 days after the end of treatment to sample various post-meiotic cells: spermatozoa (days 5–7), late spermatids (days 8–10) and mid + late spermatids (days 112118). | Increased MN in morphologically normal and abnormal embryos, respectively (41 and 93 MN per 1000 cells) |
| [144] | Mouse (pregnant female); 3-day embryo | Trichlorfon | i.p. injection at 6 h post-presumed conception and either sacrificed on day of gestation (dg) 3, 9 or 17. | Increased MN in embryos at dg 3. |
| [145] | Mouse (pregnant female); 3-day embryo (blastocysts) | Chlorpyrifos | i.p. injection on day 0 of pregnancy. Evaluated on day 3 of gestation. | Increased MN per embryo and the percentages of embryos with at least one MN. |

MMS, methyl methanesulfonate; MMC, mitomycin C; B[*a*]p, benzo[*a*]pyrene; DEN, diethylnitrosamine; MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine; DMBA, 7,12-dimethylbenz[*a*]anthracene.

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# Report of the IWGT working group on strategies and interpretation of regulatory *in vivo* tests I. Increases in micronucleated bone marrow cells in rodents that do not indicate genotoxic hazards

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**Abstract**

*In vivo* genotoxicity tests play a pivotal role in genotoxicity testing batteries. They are used both to determine if potential genotoxicity observed *in vitro* is realised *in vivo* and to detect any genotoxic carcinogens that are poorly detected *in vitro*. It is recognised that individual *in vivo* genotoxicity tests have limited sensitivity but good specificity. Thus, a positive result from the established *in vivo* assays is taken as strong evidence for genotoxic carcinogenicity of the compound tested. However, there is a growing body of evidence that compound-related disturbances in the physiology of the rodents used in these assays can result in increases in micronucleated cells in the bone marrow that are not related to the intrinsic genotoxicity of the compound under test. For rodent bone marrow or peripheral blood micronucleus tests, these disturbances include changes in core body temperature (hypothermia and hyperthermia) and increases in erythropoiesis following prior toxicity to erythroblasts or by direct stimulation of cell division in these cells. This paper reviews relevant data from the literature and also previously unpublished data obtained from a questionnaire devised by the IWGT working group. Regulatory implications of these findings are discussed and flow diagrams have been provided to aid in interpretation and decision-making when such changes in physiology are suspected.
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*Keywords:* IWGT; Genotoxicity tests; *In vivo*; Rodent bone marrow; Micronucleus; Specificity; False positive; Changes in physiology; Hypothermia; Hyperthermia; Spindle disruption; Erythropoiesis; Bone marrow cell toxicity; Pharmacologically related changes; Regulatory implications

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