Mutagenesis vol. 26 no. 1 pp. 223–230, 2011 doi:10.1093/mutage/geq066

REVIEW

Micronucleus assays in rodent tissues other than bone marrow

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Received on June 7, 2010; revised on July 26, 2010; accepted on August 25, 2010

This report updates previous reviews that were conducted as part of the third and fourth International Workshops on Genetic Toxicology Testing of micronucleus (MN) assays in rodent tissues other than bone marrow. Tissues discussed here are liver, lung, skin, colon, spleen, testes and foetal/neonatal tissues with transplacental exposure. Previous reviews have been updated to include literature published after 2000. In addition to the previously described tissues, MN assays in bladder, buccal mucosal cells, stomach and vagina are also included. MN assays using tissues other than bone marrow are critical for risk assessments, for *in situ* evaluation and for studies of systemic genotoxic effects and modes of action. Protocols for the majority of assays in tissues other than bone marrow have not yet been well standardised and validated for regulatory application, and further development is needed to support regulatory studies.

Introduction

Evaluation of genetic toxicity is an important component of the safety assessment of chemicals, including pharmaceuticals, agricultural chemicals, food additives and industrial chemicals. Up to the present time, genotoxicity has been regulated mainly on the basis of qualitative outcomes of hazard identification assays, i.e. decisions are often based on classification as positive or negative for genotoxic potential. Recently, the field is moving towards quantitative risk assessments and with more reliance on weight of evidence (WOE) approaches (1–3). *In vivo* assays are critical components of both WOE analysis and of quantitative risk assessments. The *in vivo* micronucleus (MN) assay using rodent haematopoietic cells from bone marrow or peripheral blood is widely used for the assessment of clastogenicity/aneugenicity of chemicals, both as a hazard screening assay and as a component of exposure-based risk assessments. The assay is usually incorporated into the standard genotoxicity test battery as a representative *in vivo* assay together with an *in vitro* gene mutation assay using bacteria and an *in vitro* chromosomal aberration assay using cultured mammalian cells (4). The MN assay was originally established using rodent erythrocytes as a simple method of assessing cytogenetic damage, taking advantage of two important characteristics of the haematopoietic system: (i) the ease of scoring MN in the newly formed anucleate reticulocytes derived from bone marrow and (ii) the ability to identify the newly formed erythrocytes that had completed their last division during or shortly after exposure to the test agent as those staining positive for RNA content (5,6). These important advantages initially led to an almost exclusive focus on the haematopoietic cells as the target. However, in principle, MN can be scored in any dividing cell population, and the assay was soon extended to tissues other than bone marrow.

One of the early extensions of the assay was to male germ cells, which are a key cell population for genetic studies and which undergo continuous active division (7,8). The assay was also extended to liver by stimulating regeneration and hence cell replication, by partial hepatectomy (9–12) or pretreatment with hepatotoxicants (13,14). Later, colon, skin and young rodent liver were studied extensively and shown to be suitable for MN analysis. Studies using human tissues or cell preparations include corneal cells and exfoliated cells from urinary bladder, oesophagus, cells of the nasal cavity and oral buccal mucosa (15,16). Rodent embryonic cells have also been used to evaluate effects of transplacental application of test chemicals. In addition, human lymphocytes have been used to assess cytogenetic damage in epidemiology studies of environmentally or occupationally exposed individuals, as summarised previously. The MN assay using peripheral lymphocytes or other human tissues is reviewed separately in this special issue.

In regulatory testing, the rodent erythrocyte MN assay is the most common first choice among *in vivo* assays used for subsequent testing when *in vitro* genotoxicity test(s) are found to be positive. The unscheduled DNA synthesis assay using rat liver has also been used in this situation, but its sensitivity has been questioned (17). The single-cell gel electrophoresis (comet) assay and gene mutation assays using transgenic animals are frequently considered for use as follow-up assays when *in vitro* gene mutation assay(s) are positive. It is important to evaluate cytogenetic damage in those cell populations or tissues that are the relevant sites of distribution and metabolism *in vivo*, and so bone marrow haematopoietic cells alone cannot satisfy this need.

Scientific considerations as well as the animal welfare movement, which emphasises the reduction, replacement and/or refinement of animal use (the '3Rs'), have contributed to the implementation of protocols that allow the integration of genotoxicity end points into general toxicological tests. Assays that have been shown to be appropriate for integration with repeat-dose general toxicology studies include the bone marrow and peripheral blood erythrocyte MN assays, the peripheral blood lymphocyte assay and a new MN assay method using rat liver after an extended period of repeated dosing (18). The liver is a key site of xenobiotic metabolism

and accumulation *in vivo*, and therefore the repeat-dose method, which appears to be applicable in adult rats, promises to be an important assay that could be incorporated into general toxicological studies.

MN assays using tissues other than bone marrow have previously been reviewed in conjunction with two International Workshops on Genotoxicity Testing (IWGT) (19,20) and have been discussed by Kirkland *et al.* (17,21). Here, we update the previous publications of the IWGT groups and revise the previous table of assay results to include studies published after 2000.

Literature search

The literature search was conducted by using PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez) and search terms that included 'micronucleus', 'rat', 'mouse' and specific tissue names with limitations of year and English language. The time limit was from 2000 to present, except for colon, liver and skin, in which cases the limit was set to 2005 or later because these tissues were evaluated in the third and fourth IWGT reviews (19,20). All abstracts identified in the searches were reviewed and then suitable articles were selected for further review and inclusion. Certain literature, for example publications of *in vitro* or *in vivo* studies of irradiation (e.g. X-ray, magnetic field), some MN studies on mouse models of human genetic disease or dietary deficiency and human studies were excluded. The tissues identified in which the rodent MN assays had been reported were alveolar, bladder, buccal mucosa, colon, liver, skin, spleen, stomach, vagina and foetal or neonatal tissues. No additional literature related to sperm or spermatid MN assays after 2000 was found. The identified assay results published within the above time periods are listed in Table I.

MN assays using tissues other than bone marrow

Liver

Liver is often a site of carcinogenic response, but liver cells replicate relatively slowly in adult animals. Therefore, in the early development stage of the liver MN assay, cell division was stimulated by partial hepatectomy (9–12) or treatment with hepatotoxicants (13,14). Early protocols were improved by subsequent investigators (25,59) who recognised that in rodents there is active cell division in liver soon after birth, i.e. up to 5–6 weeks in rat. Based on this characteristic, young rats (22,23,26,27,29) or mice (28) can be used for MN frequency determination without any additional physical or chemical treatment.

As noted above, recently a new protocol using adult rat liver in combination with repeated treatments is now under development (Narumi *et al.*, submitted for publication). This method should be easily integrated with general toxicological studies. As an example, adult rats that received daily *N*-nitrosodiethylamine (DEN) or 2,4-diaminotoluene (2,4-DAT) for 5, 14 and 28 days orally without any additional treatment had elevated frequencies of micronucleated hepatocytes after repeated treatments with DEN for $\geq$5 days or with 2,4-DAT for $\geq$14 days. The observed frequencies increased with the number of treatments, as expected. To verify the reliability of this method, cell proliferation in liver has been investigated with bromodeoxyuridine intake. Atrazine also induced MN in rat liver treated for 21 days (24). This method promises to allow monitoring of genetic damage in this important tissue during routine repeated-dose toxicology assays.

Colon and intestinal epithelium

The colon and digestive tract are important target sites of carcinogens (e.g. hydrazines). In addition, colon is an important tissue for metabolism by the intestinal microflora and excretion by feces (30). Therefore, the MN assay in colon and/or intestine is appropriate for hazard identification and risk evaluation of environmental chemicals that enter the human body through the food chain, such as pesticides and food ingredients, as well as for orally administrated pharmaceuticals (33). The colon MN assay with the mouse was developed by Goldberg *et al.* (60), and it has been improved by Vanhauwaert *et al.* (61) and Ohyama *et al.* (62). The colon MN assay has been used for the confirmation of the presence or absence of genotoxic activity in colon (30–33). Because cells in the colon originate from stem cells at the base of the crypts that are vascularised, it is also possible that MN in colon cells may be induced *via* blood-borne genotoxicants.

Skin

Skin is the major site of exposure to many chemicals, including industrial chemicals, pesticides, consumer products, cosmetics and pharmaceuticals. Skin is also an important tissue for protection from environmental agents, including light and chemicals. Although the use of *in vitro* photo-genotoxicity assays is diminishing because of oversensitivity and low specificity, the skin MN assay provides a more relevant method of assessing the risk of *in vivo* photo-genotoxicity associated with exposure to chemical and physical agents (35,36). The skin MN assay in the mouse was introduced around 1990 (63,64), and it was then developed in the rat and hairless mouse by Nishikawa *et al.* (34,65,66). In this assay, acridine orange fluorescent staining is playing an important role not only to identify MN but also to identify the appropriate cell population to be analyzed (34,35). Although many studies have been conducted using skin application with or without ultraviolet irradiation, detection of systemic effects in skin following oral administration is also important (67).

Spleen

The spleen MN assay with mouse splenocytes has been developed by Shindo *et al.* (68), using splenocytes as the target cell population. Evaluation of genotoxic effects in splenocytes during subchronic exposures provides another relevant target tissue in addition to haematopoietic cells in bone marrow or peripheral blood (37–40). *In vitro* and/or *in vivo* methods have been used in many studies (38,40–42).

Lung

Lung is one of the major target sites of those carcinogens for which inhalation is the major route of exposure. Therefore, lung cells are an important target site for detection of local genotoxic effects of environmental or industrial chemicals following or during inhalation exposures (43,45–47). Intratracheal instillation is also often used as a means of delivery to the lung in animal experiments (43). Studies of long-term exposure (several weeks or months) by inhalation are also possible (44,47). A lung MN assay was developed as an *in vivo*/*in vitro* assay in early 1990s (69,70), and in more recent

Table I. MN assays in multiple organs in rodents published after the search in 4th IWGT[a]

Specific tissue/organ, chemical	Species tested	Administration		Sampling time after the final treatment	MN in the specific organ	
		Route	Times per duration		Result	Refs.
Liver						
2-Acetylaminofluorene	Rat[c]	po	Twice	3, 4, 5 days	+	(22)
Allyl alchohol	Rat[c]	po	Once	3, 4, 5 days	–	(23)
Atrazine	Rat	po	7, 14, 21 days (once daily)	Not described	+	(24)
Bromobenzene	Rat[c]	po	Once	3, 4, 5 days	E[d]	(23)
Carbendazim	Mouse[e]	po	Once	4 days	+	(25)
4-Chloro-*o*-phenylenediamine	Rat[c]	ip or po	Once	3, 4, 5 days	–	(26)
Chlorpromazine hydrochloride	Rat[c]	po	Once	3, 4, 5 days	–	(23)
Clofibrate	Rat[c]	po	Once	3, 4, 5 days	–	(23)
Colchicine	Mouse[e]	iv	Once	4 days	+	(25)
Cyclophsphamide	Rat[c]	po	Once	3, 4, 5 days	+	(22)
Cytochalasin B	Mouse[e]	iv	Once	4 days	+	(25)
2,4-Diaminotoluene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,27)
Diazepam	Mouse[e]	iv	Once	4 days	+	(25)
Di(2-ethylhexyl)phthalate	Rat[c]	ip or po	Once	3, 4, 5 days	–	(26)
Diethylnitrosamine	Rat[c]	po	Once, twice	5 days	+	(22,23,26,27)
p-Dimethylaminoazobenzene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,26,27)
1,2-Dimethylhydrazine dihydrochloride	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,27)
Dimethylnitrosamine	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,26)
2,4-Dinitrotoluene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,27)
2,6-Dinitrotoluene	Rat[c]	po	Once, twice	3, 4, 5 days	+	(23,27)
1,4-Dioxane	Mouse[e]	po	5 days (once daily)	24 h	+	(28)
Ethyl methanesulfonate	Rat[c]	ip, po	Twice	3, 4, 5 days	–	(22)
5-Fluorouracil	Rat[c]	ip	Once	3, 4, 5 days	–	(22)
Isoniazid	Rat[c]	po	Once	3, 4, 5 days	–	(23)
Kojic acid	Rat[c]	ip or po	Once	3, 4, 5 days	–	(26)
4,4'-Methylenedianiline	Rat[c]	ip or po	Once	3, 4, 5 days	–	(26)
Methyl methanesulfonate	Rat[c]	ip or po	Once	3, 4, 5 days	–	(26)
Mitomycin C	Rat[c]	ip	Once, twice	3, 4, 5 days	+	(22,27)
Alpha-naphthyl isothiocyanate	Rat[c]	po	Once	3, 4, 5 days	–	(23)
Noscapin	Mouse[e]	iv	Once	4 days	+	(25)
Paclitaxel	Mouse[e]	iv	Once	4 days	+	(25)
Phenacetin	Rat[c]	po	Once	3, 4, 5 days	–	(23)
Quinoline	Rat[c]	po	Once, twice	3, 4, 5 days	+	(22,26)
Thioacetamide	Rat[c]	po	Once	3, 4, 5 days	–	(23)
o-Toluidine	Rat[c]	ip or po	Once	3, 4, 5 days	–	(26)
Trichlorfon	Mouse[e]	iv	Once	4 days	+	(25)
Vinblastine sulfate	Rat[f]	ip	Once	24 h	+[g]	(29)
Vinblastine sulfate	Mouse[e]	ip	5 days (once daily)	24 h	+	(28)
Vitamin E	Rat	po	7, 14, 21 days (once daily)	Not described	–	(24)
Colon and intestinal epithelium						
Amaranth	Mouse	po	Twice	24 h	–	(30)
Colchicine	Mouse	po	Once	24, 36, 48 h	+	(31)
Cyclohexane chlorination products	Mouse	po	4 days (once daily)	24 h	+	(32)
Lambda-cyhalothrin	Rat	po	13 days (once per 48 h)	30 h	+	(33)
Dimethylaminoazobenzene	Mouse	po	Twice	24 h	–	(30)
1,2-Dimethylhydrazine	Mouse	po	Once	24 h	+	(30,31)
Mitomycin C	Rat	ip	Once	30 h	+	(33)
Okadaic acid	Mouse	po	Once	24, 36, 48 h	Inc[h]	(31)
Sunset yellow	Mouse	po	Twice	24 h	–	(30)
Tartrazine	Mouse	po	Twice	24 h	–	(30)
Skin						
Anthracene	Mouse	sa	3 days (daily)	24 h	–	(34)
Benz[*a*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
Benzo[*a*]pyrene	Mouse	sa	Once	72, 96 h	+[i]	(35)
Benzo[*a*]pyrene	Mouse	sa	3 days (daily)	24 h	+	(34)
Benzo[*e*]pyrene	Mouse	sa	3 days (daily)	24 h	–	(34)
Chrysene	Mouse	sa	3 days (daily)	24 h	–	(34)
Dibenz[*a,c*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
Dibenz[*a,h*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
7,12-Dimethylbenz[*a*]anthracene	Mouse	sa	3 days (daily)	24 h	+	(34)
Kojic acid	Mouse	sa	Twice	48 h	–[i]	(36)
8-Methoxypsoralen	Mouse	sa	Twice, once	48 h, 24–168 h	+[i]	(35,36)
3-Methylcholanthrene	Mouse	sa	3 days (daily)	24 h	+	(34)
Mitomycin C	Mouse	sa	3 days (daily)	24 h	+	(34)
Pyrene	Mouse	sa	3 days (daily)	24 h	–	(34)

Table I. *Continued*

Specific tissue/organ, chemical	Species tested	Administration		Sampling time after the final treatment	MN in the specific organ	
		Route	Times per duration		Result	Refs.
Spleen						
Aflatoxin B1	Rat	po (diet)	90 days	End of feeding	+	(37)
Benzo[*a*]pyrene	Mouse	po	4 weeks (twice weekly)	End of exposure	+[j]	(38)
Cyclophosphamide	Mouse	ip	Once	24 h	+	(39)
Ethylene oxide	Rat	inh	4 weeks (6 h/day, 5 days/week)	5 days	–[j]	(40)
Ethylene oxide	Rat	ip	Once	24 h	+	(40)
Fumonisin B1	Rat	po (diet)	90 days	Not described	+	(37)
Furan	Mouse	po	4 weeks (5 days/week)	24 h	+[j]	(41)
Furan	Mouse	po	Once	3 h	–[j]	(41)
Hydroquinone	Mouse	ip	6 days (daily)	24 h	+[j]	(42)
Methyl methanesulfonate	Mouse	ip	Once	3, 24 h	+	(41)
N-Methylmorphine chloride	Mouse	ip	Once	24 h	+	(39)
Metyrapone	Mouse	ip	Once	24 h	–	(39)
Morphine sulfate	Mouse	ip	Once	24 h	+	(39)
Lung						
Bleomycin	Rat	it	Once	72 h	+	(43)
Dioxazid	Rat	inh	3 months	Not described	–	(44)
Environmental cigarette smoke	Mouse	inh	15 days (6 h/day)	End of exposure	+	(45)
Ethylene oxide	Mouse	inh	4 h	72 h	+	(46)
Formaldehyde	Rat	inh	4 weeks (6 h/day, 5 days/week)	End of exposure	–	(47)
WC-Co dust[b]	Rat	it	Once	72 h	+	(43)
Stomach						
N-methyl-*N'*-nitro-*N*-nitrosoguanidine	Mouse	po	Once	3, 4 days	+	(48)
N-nitroso-*N*-methylurea	Mouse	po	Once	3, 4 days	+	(48)
Bladder						
N-acetylcysteine	Mouse	ip	Once	10 days	–	(49)
Cyclohexane chlorination products	Mouse	po	4 days (daily)	24 h	+	(32)
Cyclophosphamide	Mouse	ip	Once	10 days	+	(49)
Dioxazid	Rat	inh	3 months	Not described	–	(44)
o-Phenylphenol	Rat	po (diet)	14–15 days	End of feeding	+	(50,51)
Sodium chloride	Rat	po (diet)	14 days	End of feeding	+	(51)
Sodium *o*-phenylphenol	Rat	po (diet)	15 days	End of feeding	+	(50)
Vitamin C	Mouse	ip	Once	10 days	–	(49)
Buccal mucosa						
Cadmium chloride	Mouse	po	6 weeks	Not described	+	(52)
Royal jelly	Mouse	po	6 weeks	Not described	–	(52)
Vagina						
Colchicine	Rat	ip	Once	Until three cycles of oestrus	+	(53)
Cyclophosphamide	Rat	ip	Once	Until three cycles of oestrus	+	(53)
5-Fluorouracil	Rat	va	5 days (daily)	Until three cycles of oestrus	+	(54)
20-Methylcholanthrene	Mouse	va	30 days (daily)	Not described	+	(55,56)
Metronidazole	Rat	va	5 days (daily)	Until three cycles of oestrus	+	(54)
Quercetin	Mouse	po (diet)	30 days	Not described	–	(56)
Vitamin E (alpha-tocopherol)	Mouse	po (water)	30 days	Not described	–	(56)
Foetal/neonatal tissues						
Fluconazole	Mouse[k]	tp by ip	3 days (PGDs 12–14, daily)	6–18 h after delivery	+	(57)
Cyclophosphamide	Mouse[k]	tp by ip	3 days (PGDs 12–14, daily)	6–18 h after delivery	–	(57)
Sodium dichromate dihydrate	Mouse[l]	tp by po (water)	18 days (PGDs 1–18)	End of feeding	–	(58)
Sodium dichromate dihydrate	Mouse[l]	tp by ip	Once (PGD 17)	1 day	+	(58)
Potassium dichromate	Mouse[l]	tp by po (water)	18 days (PGDs 1–18)	End of feeding	–	(58)
Potassium dichromate	Mouse[l]	tp by ip	Once (PGD 17)	1 day	+	(58)

inh, inhalation; ip, intraperitoneal injection; it, intratracheal instillation; po, per os; va, vaginal application; sa, skin application; tp, transplacental; PGD, pregnancy day; Inc, inconclusive; E, equivocal.

[a]For colon and intestinal epithelium, liver and skin, published data from 2005 to present. For lung, bladder, buccal mucosa, spleen, stomach, vagina and foetal/neonatal tissues, published data from 2000 to present.

[b]Consisted of 6.3% cobalt, 84% tangsten and 5.4% carbon.

[c]4-Week-old.

[d]Positive at toxic dose.

[e]Partial hepatectomy.

[f]5-Day-old.

[g]Erythrocytes from liver.

[h]Both positive and negative results.

[i]With light irradiation.

[j]*In vivo/in vitro.*

[k]Peripheral blood from neonatal 6–18 h after delivery.

[l]Peripheral blood and liver from foetus on PGD 18.

studies, several types of lung cells have been used, including bronchoalveolar lavage cells, alveolar type II cells, Clara cells or pulmonary alveolar macrophages (46,47).

Stomach

Stomach is important because it is the first site of direct contact of many chemicals. Several *in vivo* genotoxicity assays using stomach have been developed, including the unscheduled DNA synthesis assay, comet assay and transgenic mutation assay. Recently, a stomach MN assay has been established by Okada *et al.* (48). Though data obtained are at present limited to two known stomach carcinogens (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and *N*-nitroso-*N*-methylurea), the assay appears promising for further development.

Bladder

Urinary bladder is a tumour target site of certain chemical classes such as naphthylamines, benzidines or aminobiphenyls. Because it is the tissue that collects urine excreted by kidneys, it will be exposed to water-soluble chemicals excreted *via* the urine. A bladder MN assay has been developed as a tool for studies of cancer risk, including intervention studies, in humans (71). Exfoliated or epithelial cells from urinary bladder have also been used in animal studies in which acute or subchronic exposures by several routes of administration were employed (32,44,49–51). The bladder MN assay is a good biomonitoring tool for humans exposed to carcinogenic or genotoxic chemicals, and analogous studies with animals can be used for identification and/or confirmation of genotoxic action in the bladder.

Buccal mucosa

Buccal mucosal cells have been used for biomonitoring of DNA damage potentially leading to oral cancer in humans (15,72). Although many studies have been conducted in humans, animal data are scant (52) or limited to animal models of human disease such as Alzheimer's disease (73). This may indicate that analysis of MN in buccal mucosal cells in rodents may not be suitable at present for detection of genotoxic effects and that technical development will be needed for general use of the detection of local genotoxic effects at this site in animals.

Vagina

Carcinoma of the uterine cervix is important in humans and has been shown to be inducible by chemical exposure in the mouse (55). A vaginal MN assay could be a useful tool for screening new chemopreventive agents in the rodent. Chakrabarti and Dutta (74) described a vaginal MN assay that could detect known MN inducers, but there is not yet sufficient data to evaluate the utility of this model (53–56).

Foetal/neonatal tissues

Assays using MN induction to evaluate transplacental genotoxicity were developed around 1980 (75–77) and many studies were published before 2000. Those studies have been summarised in previous IWGT reports (19,20). In the late 1980s, MacGregor *et al.* (78,79) showed that additional kinetic information could be obtained in the foetal blood MN assay by categorising newly formed red blood cells into age groups based on RNA aggregation state. Additional data from studies using peripheral blood from neonates or peripheral blood and liver from foetuses, found in our search of the literature published after 2005 are summarised in Table I (57,58). Foetal/neonatal MN assays using transplacental exposure will be useful for evaluation of agents associated with embryotoxicity, congenital malformations, tumours, heritable mutations in germ cells or other adverse developmental effects.

Spermatogonia/spermatids

A spermatogonial MN assay was proposed in the early 1980s (7,8), and early results of this assay were summarised in the previous IWGT reports (19,20). No new data were identified in our PubMed search. Initially, it was difficult to identify the specific developmental stage of spermatogonial cells and to restrict the cell population to be analysed. A fluorescent staining method using acridine orange and 4',6-diamino-2-phenylindole concomitantly has now been introduced and it facilitates identification of the spermatogonial cells to be analysed (80). This staining method has also been applied to other tissues successfully because the cytoplasm and nucleus are clearly stained and provide increased accuracy of scoring micronucleated cells.

The evaluation of MN in reproductive tissue is obviously important in the evaluation of adverse effects to subsequent generations. The United Nations (UN) 'Globally Harmonised System of Classification and Labelling of Chemicals (GHS)' requests hazard classification of chemicals based on germ cell mutagenicity (81,82) and identifies the spermatid MN assay as an example of a mutagenicity test applicable to germ cells.

Discussion

Many tissues other than bone marrow or peripheral blood have been used for evaluation of MN frequencies *in vivo* in rodents. In our literature search, liver, colon epithelium, skin, spleen, lung, stomach, bladder, buccal mucosa, vagina and foetal/neonatal tissues were identified as target tissues used for such assays. No new data on MN incidence in spermatogonia or spermatids were identified after the previous IWGT reviews (19,20). These MN assays have been used to detect local or systemic genotoxic effect in important tissues, including germ cells and progeny.

These assays are important tools for the prediction and evaluation of chemical carcinogenicity and also for the identification and study of agents with the potential to induce transmissible germ line mutations. Currently, there is emphasis on extending hazard identification techniques to evaluate genotoxic risk more quantitatively (1,2,83). One important objective of genotoxicity evaluation is the assessment of the mechanism of carcinogenicity, and whether genotoxic mechanisms are involved or not. Another is to assess adverse heritable effects of chemicals that may appear in subsequent generations. For the former purpose, assays are needed that permit assessment in the appropriate target tissues and allow development of mode of action (MOA) and a WOE of information in conjunction with information about exposure and metabolic transformation (84). The tissues with most active cell division are bone marrow, gastrointestinal tract, skin and reproductive tissues in mammals. These tissues have been used as experimental materials for the analysis of chromosomal damage, including evaluation of induction of chromosomal aberrations, and the ability to monitor MN frequencies in these same tissues is therefore important.

Given the recent emphasis on genotoxic risk characterisation, MOA determination and WOE approaches (84), the ability to study a wide range of tissues will become increasingly important. However, validated standard protocols

(e.g. Organization for Economic Co-operation and Development test guidelines) have not yet been established for most of these tissues, and methods remain primarily research oriented. Even liver, colon, skin and spermatogonia/spermatid MN assays, which have been used relatively frequently for research purposes, are still infrequently used for regulatory purposes. The target cell populations, cell preparation, suitable sampling time(s), cytotoxicity evaluation (dose selection) and/or historical background data are less well established in other tissues, such as lung, bladder, buccal mucosa, nasal epithelium and vagina. Investigation of cell division is a critical issue when studying MN in any tissue. Suitable sampling time(s) after the final treatment varies in different tissues or study designs including the parameters of treatment duration or route of exposure. Further development, especially to establish standard protocols and validation of these MN assays, is highly desirable and will facilitate their increasing application to support more meaningful and relevant assessments of clastogenic/aneugenic risks associated with chemical and physical exposures (1,2,17,21,83).

Funding

Health and Labor Sciences Research Grants (H21-Chemistry-General-002).

Acknowledgements

Conflict of interest statement: None declared.

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Mutation Research xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Workshop summary: Top concentration for in vitro mammalian cell genotoxicity assays; and report from working group on toxicity measures and top concentration for in vitro cytogenetics assays (chromosome aberrations and micronucleus)

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ARTICLE INFO

Article history:
Received 12 January 2011
Accepted 12 January 2011
Available online xxx

Key words:
In vitro tests
Chromosome aberrations
Micronucleus
Cytotoxicity
Upper concentration limit

ABSTRACT

The selection of maximum concentrations for in vitro mammalian cell genotoxicity assays was reviewed at the 5th International Workshop on Genotoxicity Testing (IWGT), 2009. Currently, the top concentration recommended when toxicity is not limiting is 10 mM or 5 mg/ml, whichever is lower. The discussion was whether to reduce the limit, and if so whether the 1 mM limit proposed for human pharmaceuticals was appropriate for testing other chemicals. The consensus was that there was reason to consider reducing the 10 mM limit, and many, but not all, attendees favored a reduction to 1 mM. Several proposals are described here for the concentration limit.

The in vitro cytogenetics expert working group also discussed appropriate measures and level of cytotoxicity. Data were reviewed from a multi-laboratory trial of the in vitro micronucleus (MN) assay with multiple cell types and several types of toxicity measurements. The group agreed on a preference for toxicity measures that take cell proliferation after the beginning of treatment into account (relative increase in cell counts, relative population doubling, cytokinesis block proliferation index or replicative index), and that this applies both to in vitro MN assays and to in vitro chromosome aberration assays. Since relative cell counts (RCC) underestimate toxicity, many group members favored making a recommendation against the use of RCC as a toxicity measure for concentration selection.

All 14 chemicals assayed for MN induction in the multi-laboratory trial were detected without exceeding 50% toxicity by any measure, but some were positive only at concentrations with toxicity quite close to 50%. The expert working group agreed to accept the cytotoxicity range recommended by OECD guideline 487 (55 ± 5% toxicity at the top concentration scored). This also reinforces the original intent of the guidance for the in vitro chromosome aberration assay, where ">50%" was intended to target the range close to 50% toxicity.

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doi:10.1016/j.mrgentox.2011.01.003

1. Upper concentration limit for in vitro mammalian cell genotoxicity assays

1.1. Introduction

At the 5th International Workshop on Genotoxicity Testing (IWGT) held in Basel, Switzerland in August 2009, one of the major topics under review was the selection of maximum concentrations for in vitro mammalian cell genotoxicity assays. Since the early 1990s, when the OECD guidelines for in vitro mammalian cell genotoxicity assays were developed, considerable experience with these assays has been gained and various analyses of their performance singly and in batteries of tests have been made (e.g., [1–3]). These large retrospective analyses of data bases indicate that the sensitivity of the in vitro mammalian cell genotoxicity tests as currently used is high, but the specificity is low due to positive results for chemicals that are not rodent carcinogens or in vivo genotoxins. Various proposals have been made by expert working groups to reconsider the top concentration used in in vitro mammalian cell genotoxicity assays as a way to increase the accuracy of in vitro testing, with an emphasis on reducing the need for in vivo follow-up testing. Recently, these include recommendations by an ECVAM[3] initiative [4], and in proposed revisions to the ICH S2 guidelines for human pharmaceuticals [5]. Currently, the top concentration recommended for in vitro mammalian cell assays when toxicity is not limiting (e.g., in OECD guidelines) is 10 mM or 5 mg/ml, whichever is lower [6,7]. One topic for the IWGT workshop was to review whether the top concentration limit of 10 mM should be lowered and if so whether a 1 mM limit as proposed in the draft revised ICH S2 guideline for pharmaceuticals [5] was appropriate for testing other types of chemicals. Because this topic was relevant both to the in vitro cytogenetic assays and the mouse lymphoma assay, it was introduced in the first plenary session of the IWGT meeting. The various analyses and proposals on this topic are reviewed below. An account of the discussion of the in vitro cytogenetic assay working group is found in their report below.

The in vitro cytogenetic working group also discussed the appropriate measures and level of cytotoxicity for in vitro cytogenetic assays. A variety of approaches to measurement of cytotoxicity have evolved over the years, which can lead to differences in test agent concentration selection. This topic was addressed in the development of the recent OECD guideline 487 for the in vitro micronucleus (MN) assay, leading to an international trial comparing various measures of cytotoxicity. The initial results of this exercise were reviewed by the IWGT in vitro cytogenetic assays working group who discussed acceptable measures and limits of toxicity. The reports from this OECD trial have since been published [8] and the OECD guideline is now final [9].

2. Upper concentration limit for in vitro mammalian cell genotoxicity assays in the absence of limiting toxicity

2.1. Plenary session. Outline of topics discussed.

2.1.1. ECVAM-sponsored review of maximum concentration in genotoxicty tests of rodent carcinogens

Dr. Raffaella Corvi summarized the results of an evaluation conducted for ECVAM by herself and Drs. James and Elizabeth Parry, in which the published genotoxicity data for mammalian cell assays were examined for 553 rodent carcinogens from the Gold Berkeley Carcinogenic Potency database [10,11]. The objective of the study was to determine how often carcinogenic chemicals gave positive results in selected concentration ranges, e.g., >1–10 mM, ≤1 mM. The authors also examined the Ames test results for chemicals in these various categories to assess the overall results for the chemical in a standard test battery. The summary of this analysis, and an overview of the initial results by Dr. David Kirkland, had been circulated to the expert working groups before the IWGT meeting. The complete report and a follow-up study are now published [10,11]. The key conclusions reported were as follows:

- Of 553 rodent carcinogens in the Gold database, in vitro genotoxicity data that met the study criteria were available for 384. Based on the authors' classification, only 24 carcinogens that were negative in the Ames test were reported as positive in the in vitro cytogenetic assay or mouse lymphoma cell *tk* mutation assay only at concentrations above 1 mM but not exceeding 10 mM (about 6%). Thus, the majority of rodent carcinogens that were positive in the in vitro mammalian cell tests and negative in the Ames test were positive in the in vitro cytogenetic/mouse lymphoma assays at 1 mM or below.[4] The 24 Ames-test-negative rodent carcinogens that were identified as positive only at >1–10 mM in the analysis by Parry et al. [10] were reviewed to assess the weight of evidence available on whether they were likely to have a genotoxic mode of action [11]. Of these 24, it was concluded that 10 chemicals were likely to be carcinogenic by a non-genotoxic mode of action, and 9 potentially had a genotoxic component to their mode of action, or a genotoxic component could not be ruled out. Five carcinogens had an unknown mode of action.
- From the 14 rodent carcinogens that might possibly have a genotoxic component to their mode of action (despite a negative Ames test) and which were genotoxic only at >1–10 mM in mammalian cell assays in vitro, 9 were selected as priorities for re-testing under protocols compliant with current guidelines and standards [11]. One further chemical, diaminozide, was tested because it was reportedly positive at >10 mM, with no data available between 1 and 10 mM. At the time of the IWGT workshop it was already known from some of these re-tests that the original positive reports could not be confirmed; the data for these re-tests, and the review of the "mode of action" data are now published [11]. Of the 10 chemicals that were retested, 5 (benzofuran, monuron, daminozide, 2-mercaptobenzothiazole and toluene) were negative either at the limit of toxicity or at concentrations up to 10 mM. Four of the others (allyl isovalerate, caffeic acid, chlorobenzene and furan) were positive at concentrations <1 mM. Only methylolacrylamide required a higher concentration for detection of a positive response, 2 mM, which, because of the low molecular weight, equated to 202 μg/ml. This re-testing experience demonstrates the improved accuracy of testing when appropriate test protocols are used (a point also emphasized in the mouse lymphoma cell mutation assay working group, elsewhere in this issue).

Overall, it was seen that only a small portion of chemicals that are rodent carcinogens and negative in the Ames test but positive in the in vitro mammalian cell assays are positive only at concentrations above 1 mM, and that many of these rodent carcinogens are likely to operate through a non-genotoxic mode of action. Such

[3] ECVAM is the European Centre for the Validation of Alternative Methods.

[4] In discussion after the meeting, a working group member noted that the actual percentage of chemicals that were negative in the battery would be much greater if the study authors had not selected the lowest concentration reported positive in any mammalian cell assay, (e.g., a result might be positive in the mouse lymphoma assay at < 1 mM, but in the chromosome aberration assay at >1 mM), or accepted positive results in only one type of assay; but the working group member concurred with the general agreement of the workshop, that the majority of rodent carcinogens that were negative in the Ames test were positive in the in vitro mammalian cell assays at 1 mM or below.

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chemicals were considered by the authors of these studies likely to represent a low risk of mutagenicity for people under normal exposure conditions.

2.1.2. ICH guideline for testing pharmaceuticals

For human pharmaceuticals, the proposed revisions to the international test guidelines [5] recommend a reduction in top concentration in the in vitro mammalian cell assays to 1 mM, when not limited by solubility or toxicity. Dr. L. Müller briefly reviewed the rationale for this recommendation, which is based on several factors including experience with pharmaceuticals that are uniquely positive in the in vitro mammalian cell assays, where the weight of evidence, based on mechanistic studies in vitro and/or negative results in appropriate in vivo studies, shows that many of the positive results observed at concentrations above 1 mM are not relevant to in vivo conditions and human exposure. The 1 mM limit also takes into account the fact that human exposures to pharmaceuticals, in circulating blood and in tissues, are much lower than 1 mM, even for compounds that accumulate in specific organs. The pharmacologically active concentrations for drugs, and the optimal substrate concentrations for many enzymes including P450s, are typically below 10 μg/ml (20 μM for molecular weight of 500). Data were summarized from information compiled in [12] on human exposure levels for drugs, including those given at high doses, and those known to accumulate in certain tissues. Human peak exposure to pharmaceuticals is generally below 10–50 μM, with exceptions such as some antibiotics, antivirals and antitumor drugs. 17% of 313 marketed pharmaceuticals had a peak exposure (Cmax) over about 50 μM, but all below 1 mM except for the unusual drug lithium (atomic weight 6.9). An important piece of information was that even drugs known to accumulate in tissues to levels 10- to 20-fold higher than plasma levels do not generally reach very high (mM) concentrations in tissues. For example, fluoxetine, a lipophilic drug with a long half-life, has a brain/plasma ratio of 20:1 but accumulates in the brain to ~10 μg/ml (~35 μM), illustrating the finding that known drugs do not have both high plasma levels and 10–20-fold accumulation in tissue.

Typically the molecular weight of pharmaceuticals is relatively high, e.g., 400, so 10 mM represents a concentration in the range of 4 mg/ml. Subsequent to the workshop, an additional notation has been made in note 7 of the ICHS2(R1) guideline, stating: "For pharmaceuticals with unusually low molecular weight (e.g., less than 200) higher test concentrations should be considered."

The current IWGT expert working group considered whether a recommendation to reduce the upper limit for in vitro cytogenetic assays was appropriate for other chemicals (besides human pharmaceuticals), and specifically whether 1 mM was suitable.

2.1.3. Proposal to use a maximum concentration in mg/ml, instead of one based on molarity

An alternative approach to a 1 mM limit was provided to the IWGT group before the meeting by Dr. Bhaskar Gollapudi who was unable to attend. This proposal addressed the concern that a mM limit represented different concentrations for different chemicals when expressed as mg/ml, since molecular weights cover a wide range. [The ability of a chemical to react with DNA is expected to be related to the number of molecules present, i.e., the molarity, so molarity should be the appropriate way to choose test concentrations, but some investigators commonly express concentrations in mg/ml.] Dr. Gollapudi considered that although thinking of test concentrations as a multiple of human exposure might be a practical approach for pharmaceuticals where exposure is known, it was not practical when such human exposure information is not available, and he suggested instead an empirical approach based on the maximum dose recommended by OECD for conducting chronic/subchronic in vivo toxicity studies, a limit dose of 1000 mg/kg/day. If 1000 mg/kg were considered simplistically as equivalent to 1000 mg/l, this in vivo limit concentration could be used as the basis for setting a similar limit concentration for use in vitro of 1000 mg/l, i.e., 1000 μg/ml. The maximum test concentration he proposed was either 10 mM or 1000/MW, whichever is lower. For a chemical with a MW of 25 the concentration tested would be 10 mM, and for one with a MW of 250 the top concentration tested would be 4 mM. This alternative proposal was thus to use a top concentration in vitro of 1000 μg/ml or 10 mM, whichever is lower.

3. Discussion of the upper concentration limit for the in vitro cytogenetics assays

3.1. Report of the in vitro cytogenetic assays working group

In the introduction to the working group session, the history behind the current regulatory guidelines was reviewed. The top concentration recommended for in vitro mammalian cell genotoxicity assays, when toxicity and solubility are not limiting, is 10 mM (e.g. [6]). This recommendation was made, for example, by expert groups developing the OECD guideline and in IWGTP reports [13,14] based on several factors, including: The need to introduce an upper limit to avoid results confounded by high osmolality; the observation that certain mutagens were detectable in the in vitro chromosome aberration assay only at quite high concentrations (between 1 and 10 mM); and a review by Ishidate of lowest effective concentrations in vitro (in an ICPEMC report [15]). In the ICPEMC report, compounds that were positive in vivo in cytogenetic tests were examined to determine what concentrations were required for their detection in vitro in mammalian cell assays, and this was part of the rationale for the 10 mM limit. However, the requirement for 10 mM to obtain positive results viewed the in vitro mammalian cell tests in isolation and not as part of a battery including the Ames test, a component of all test batteries.

3.1.1. Re-examination of the ICPEMC report

A summary statement was made at this IWGT workshop that a re-examination of the data in the report referred to above (Fig. 1 in [15]) shows that of the in vivo positive chemicals, almost all were either detected in the Ames test, or in the in vitro chromosome aberration assay below 1 mM. The exceptions were barbital (which causes kidney tumors in male rats, likely by a non-genotoxic mechanism), and urethane, which is well known as an in vivo genotoxin that is missed by the standard in vitro battery.

3.1.2. Review of in vitro chromosome aberration data in CHL cells

The group also considered a data set from Japan, on results from high production volume chemicals, compiled and presented at the IWGT meeting by Dr. Takeshi Morita. This analysis reviewed in vitro chromosome aberration data that had been generated from 1994 to 2006, in CHL cells. It is recognized that studies prior to 1997 were from protocols that did not follow the OECD guideline published in 1997; the data set was modified to eliminate those that were positive only with a 48 h continuous treatment time that is no longer used. Of 249 chemicals, 45% (113 chemicals) were positive in the in vitro chromosome aberration assay, confirming the high frequency of positive results in this test system. Three compounds were eliminated from analysis because they were positive only at >10 mM, so they would not be detected even by the current 10 mM limit. Of the remaining 110 positive chemicals, 59 were positive for chromosomal aberrations at ≤1 mM and 51 at >1 mM. Of these 51, 14 were positive in the Ames test. Thus, there were 37 chemicals that were positive for chromosome aberrations between 1 and 10 mM that did not give positive results in the Ames test. Of these 37 chemicals, 20 were considered to be of low concern (Table 1).

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Table 1
Evaluation of data produced in a Japanese study assessing high volume chemicals for in vitro chromosome aberration induction in CHL cells. Shown are conclusions on the relevance of the responses for 37 chemicals that were positive in the assay at concentrations >1 mM and ≤10 mM, and negative in the Ames test. Evaluation conducted by T. Morita (unpublished).

Relevance	Reason for relevance conclusion	Number	Number negative /number tested in vivo in bone marrow micronucleus assay
"Irrelevant" positive (20)	Low pH	5	2/2
	High toxicity	13	6/6
	In vitro polyploid only	2	1/1
Unknown relevance (17)	Not defined	17[a]	2/2[a]

[a] Eight of these 17 chemicals were positive only with S-9 in the in vitro chromosome aberration assay in CHL cells. Two of these 8 were also negative in the in vivo bone marrow micronucleus assay.

Dr. Morita concluded that the percentage of chemicals that may be "relevant" as a human health risk but are negative in the Ames test and positive in the in vitro chromosome aberration assay only above 1 mM is quite low (17 of the 113 in vitro aberration positive chemicals, or 15%). Further review of the nature of these chemicals is on-going (Morita, in preparation), so the significance of these findings is not known at present.

3.1.3. Discussion of the in vitro cytogenetics working group on the upper concentration limit

The consensus among the working group was that there was evidence supporting the reduction of the upper concentration limit for in vitro cytogenetics assays below 10 mM without significant loss of sensitivity. It was agreed that 1 mM was appropriate for the majority of chemicals in the data sets reviewed, and many members of the group considered that 1 mM could now be recommended as a suitable limit, but this was not a consensus position. One suggestion was that 1–2 mM might be suitable for screening but that higher concentrations might be needed for certain situations. Discussion centered on the possibility of using a 1 mM limit but defining guidance on exceptions, and covered the following points:

a. Inadequate metabolism. It was recognized that some of the chemicals detectable only above 1 mM reflect poor metabolism, for example the requirement for Cyp2E1 which is poorly represented in activation systems such as rat liver S-9 and is needed to activate some chemicals including certain nitrosamines. It was pointed out that inadequate in vitro metabolism should not be a reason to test all chemicals at higher concentrations; it is also known that at high concentrations different metabolic processes may be involved, for example involving P450s that would not normally act on the substrate in question, and producing metabolites that may induce genotoxicity for the wrong reasons; this issue is discussed in more detail in reports of the IWGT [16] and EVCAM [4].
b. Complex mixtures. It was recognized that for complex mixtures a limit expressed in molarity was not practical since molecular weights are not known or are mixed. It was suggested that for complex mixtures, a higher limit such as 5 mg/ml could be maintained, based on an examination by D. Marzin of a set of 20 mixtures; these are addressed in the report of the working group on the mouse lymphoma cell mutation assay in this issue.
c. Impurities. Testing technical grade materials for "qualification" of impurities.
d. Specific chemical classes which exhibit structural alerts for genotoxic carcinogenicity (low molecular weight aldehydes were suggested).
e. Volatility. Test agent volatility is a recognized issue for in vitro assays in general, especially with lower molecular weight chemicals. The group agreed that like metabolism, this illustrates the need for improved test design, not a high concentration limit.
f. High exposure expected (unusually high circulating blood levels, or high exposure at specific localized sites). It was noted that for ethanol, for example, plasma concentrations exceeding 1 mM can occur, and high exposure to certain food ingredients is also known. (Dermal exposure is the subject of a separate concurrent IWGT working group discussion at this workshop [see Pfuhler et al., this issue].)

In discussing how to determine the appropriate conditions for in vitro testing, the overall approach of using rodent carcinogenicity assays as the sole standard against which the performance of short term tests for genetic toxicity is measured was questioned by some workgroup members. Because of the very brief time available, the group agreed that the topic of how to determine suitable top concentrations required further discussion.

4. Final plenary session: conclusions on the upper concentration limit

In the final follow-up plenary session, there was further discussion of upper concentration limits for in vitro mammalian cell assays on chemicals other than human pharmaceuticals. There was consensus that there was reason to consider reducing the 10 mM limit, and many attendees favored a reduction to 1 mM. However, there was not agreement on what the upper concentration limit should be. Several alternative proposals were put forward. The first listed below, (a), had been discussed by the in vitro cytogenetics working group, but the others had not been discussed in detail to determine whether the same exceptions would apply.

a. 1 mM may be acceptable for routine testing
   Exceptions proposed included low molecular weight substances, certain chemicals with high (particularly local) exposure, complex mixtures, and technical grade materials tested to assess impurities.
b. 2 mM may be acceptable for routine testing
   Similar exceptions to above may apply
c. 1 mM or 500 μg/ml, whichever is the higher, may be acceptable
d. A top concentration of 1000 μg/ml or 10 mM, whichever is the lower, may be acceptable
   In practice, this would mean that low MW chemicals would be tested to 10 mM but many chemicals would be tested to about 2–4 mM.

5. Appropriate measures and level of cytotoxicity for in vitro cytogenetic assays

5.1. Report of the in vitro cytogenetic assays working group

In vitro cytogenetic assays have been used for decades. In early testing, concentrations were often limited only by the ability to recover sufficient cells to score. Since then several approaches to the measurement of cytotoxicity have evolved, often with more precise analyses that take into account cell proliferation/growth. Differences in the way in which toxicity is measured lead to differences in concentration selection and assay acceptability, and have been discussed extensively [17–21]. This topic arose during the development of the recent OECD guideline for the in vitro MN

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Table 2
Common measures of cytotoxicity used for in vitro cytogenetic assays.

Measure (defined below)	Abbreviation (cf control)	Chromosome aberration assay	Micronucleus assay	Measure provides indication of proliferation
Mitotic index	RMI (relative mitotic index)	√		Yes
Binucleate cell index with cytochalasin B/replicative index	CBPI/RI (cytokinesis block proliferation index/replicative index)		√	Yes
Population doubling (without cytochalsin B in micronucleus assay)	RPD (relative population doubling)	√	√	Yes
Increase in cell counts (without cytochalsin B in micronucleus assay)	RICC (relative increase in cell count)	√	√	Yes
Cell counts (without cytochalsin B in micronucleus assay)	RCC (relative cell count)	√	√	No

Definitions: calculations of toxicity measures (usually expressed as percentages):

RMI	[No. mitotic cells/total No. cells (*treated*)]/[No. mitotic cells/total No. cells (*control*)]
CBPI	([(No. mononucleate cells) + (2 × No. binucleate cells) + (3 × No. multinucleate cells)])/(Total No. cells)
RI	([(No. binucleate cells) + (2 × No. multinucleate cells) cells)]/(Total No. cells) *treated*)/([(No. binucleate cells) + (2 × No. multinucleate cells)]/(Total No. cells) *control*)
RICC	[Final No. cells – initial No. cells (*treated*)]/[Final No. cells – initial No. cells (*control*)]
RPD[a]	(No. of Population doublings *treated*)/(No. of population doublings *control*)

No., number.
[a] Population Doubling = [log(final cell number/initial cell number)]/log 2.

assay [9], leading to a multi-laboratory, international trial to compare various approaches for measuring cytotoxicity. The results of this study were reviewed at the IWGT meeting and have since been published (summarized in [8,22]); there was overall agreement on using measurements based on cell proliferation for cytogenetic assays. The results of the IWGT working group discussions are reported below. For the purposes of this meeting, cytogenetic assays were defined as both types of in vitro chromosome damage assays: chromosome aberration assays where cells are analyzed at metaphase, and MN assays conducted either with or without Cytochalasin B (CytoB).

5.1.1. Review of measures of cytotoxicity

It is recognized that a balance is required in choosing toxicity limits to obtain appropriate sensitivity and specificity of in vitro mammalian cell testing. To help ensure sensitivity, high concentrations of a test article are used, but excessive toxicity can result in "false" negative results if cell growth is delayed so that they do not reach metaphase, or they fail to progress through metaphase into the next interphase where micronuclei can be detected. On the other hand, excessive toxicity can give rise to "misleading" positive results that occur only under cytotoxic conditions and not at lower concentrations. They are considered misleading because the events that lead to chromosome aberrations originate directly or indirectly from the processes that result in toxicity and that do not operate at lower concentrations relevant to normal usage or to human exposure; thus the dose response is expected to be non-linear.

The measures of cytotoxicity that are commonly used (Table 2) were reviewed by the in vitro cytogenetic working group. As indicated in Table 2, the relative cell count (RCC) is a measure of cytotoxicity that does not provide information on cell proliferation. An example is provided to demonstrate how RCC can underestimate toxicity (Table 3). (For simplicity, this example shows results after only one doubling in the controls.) In this example, cell numbers in the treated population did not increase during the assay (either because there was no cell proliferation, or cell proliferation was balanced by cell death), whereas the control population doubled in cell number between the beginning of treatment and the harvest, as expected. The calculation of relative population doubling (RPD) and relative increase in cell count (RICC), in this case zero, reflects the fact that there is no increase in the population of cells, i.e. the treated culture experiences 100% toxicity relative to the increase observed in the control culture. The calculation of RCC, which is based on comparing the cell counts only at the end of the assay, indicates 50% fewer cells in the treated culture compared to the control, or only 50% toxicity. In this example, toxicity is greatly underestimated when calculated by RCC compared to RPD and RICC.

Table 3
An example comparing the effects of using RPD/RICC or RCC to estimate cytotoxicity.

Culture	Initial cell number at treatment start (million)	Final cell number (million)	% toxicity (by RPD or RICC)	% toxicity (by RCC)
Control	0.5	1.0		
Treated	0.5	0.5	100% (no growth)	50% (50%)

Because micronuclei can only be observed in cells that have gone through mitosis, the expert working group affirmed the conclusion reached previously by various investigators and expert groups [18–21,23,24], i.e., that toxicity measures that provide information on the amount of cell proliferation that occurs after the beginning of treatment are preferred for measuring toxicity in cytogenetic assays.

5.1.1.1. Review of data generated using different measures of cytotoxicity. Data were reviewed from collaborative studies conducted on 14 chemicals in 12 laboratories as part of the process for drafting OECD guideline 487 for the in vitro MN assay. The results were summarized at the IWGT meeting by Kirkland and Schuler, and details for some of the chemicals were presented. The data are in individual reports in a special issue of *Mutation Research*, with a summary and conclusions by Kirkland [8,22]. The conclusions are summarized here.

These studies assessed:

- Multiple toxicity measures concurrently
- Five cell lines: L5178Y mouse lymphoma cells; TK6 human lymphoblastoid cells; and three hamster cell lines, CHO, CHL and V79.
- 14 clastogens and aneugens of various chemical classes/mechanisms, including strong and weak genotoxins: aminoanthracene, benzo[*a*]pyrene; cadmium chloride; colchicine; cyclophosphamide; cytosine arabinoside; diazepam; diethylstilboestrol; etoposide; 5-fluorouracil; mitomycin C; phenolphthalein; quinacrine and vinblastine.

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The studies followed the draft OECD guideline 487 for the in vitro MN assay and each chemical was tested in at least 2 laboratories and in as many cell types as possible. (The OECD guideline has since been published as a final guideline [9].) Not every chemical was tested in every cell type. Every laboratory conducted tests in the absence of CytoB, but some laboratories also conducted tests in the presence of CytoB so that the cytokinesis block proliferation index [CBPI] and replication index [RI] could be compared with measures used for assays conducted in the absence of CytoB (i.e., RCC, RICC, and RPD). For all of the chemicals, the method used for estimating toxicity affected the concentrations selected for scoring. For all chemicals and for each cell type, either the extent of toxicity according to RCC at a given concentration was less than that according to RICC, or the concentration required to achieve a particular level of cytotoxicity was higher in the case of RCC than for RICC. Thus RICC never identified a higher concentration target range for testing than did RCC.

The results indicate that all of the measures of cytotoxicity (RICC, RPD, CBPI/RI and RCC) allowed detection of all the chemicals (both weak and potent chemicals) at or below an upper limit of 55 ± 5% toxicity, as recommended in OECD guideline 487. In the presence of CytoB there was one exception, quinacrine, which did not give a significant increase in micronuclei with 56% suppression of proliferation (CBPI); this may be related to the well known very narrow active concentration range for detecting positive responses with aneugens (see for example [25]). Increases in micronuclei were seen with quinacrine when cytoB was not used [26].

It was noted that good study design may require closely spaced concentrations (much closer than half-log or doubling dilutions) particularly for aneugens, some nucleoside analogues and other test agents with steep toxicity dose relationships.

The use of CytoB was also discussed briefly. CytoB prevents cell division following nuclear division, resulting in binucleated cells. Thus micronuclei can be scored in interphase cells that are clearly identified as having recently divided. In some cases however, micronuclei are observed in mono-nucleated cells in preparations treated with CytoB, and the results of the collaborative study on 14 chemicals (above) reaffirmed that when increases in micronuclei are concurrently noted in mononucleated cells, they should be scored (a; see below). It was also noted that based on experience with compounds that interfere with mitosis, when cells with asymmetrical multinucleated morphology are seen (b; see below), they can complicate scoring and indicate the need to repeat the assay without CytoB (see for example [26]). Asymmetrical multinucleated morphology has been seen for example with phenolphthalein, and with "reference" aneugens such as taxol, colchicine and vincristine.

The group recommended that if equivocal results are found in an in vitro MN assay in either of these two situations (a or b in the preceding paragraph), a repeat test without CytoB is needed. It was noted by M. Schuler that in certain circumstances CytoB can increase the toxicity of a test chemical, examples being diazepam, or the increase in apoptosis with CytoB and phenolphthalein.

5.1.1.2. Conclusions on measures of cytotoxicity. Taking into consideration the principle illustrated in Table 3, and the results of the comparison of the various measures of cytotoxicity in the collaborative MN trial, the expert working group agreed on a preference for using methods for measuring toxicity that take cell proliferation after the beginning of treatment into account (RICC, RPD, CBPI/RI). Further, since RCC underestimates toxicity, many group members favored making a recommendation against the use of RCC as a toxicity measure for concentration selection. This was not a consensus position however, and it was noted that an ongoing study (Fowler et al., in preparation) will provide further information on RCC. (RCC is not one of the recommended toxicity measures in the OECD guideline for in vitro MN testing [9].) This data collection and review by Fowler and others is an examination of chemicals that give "misleading positive" results (Ames test-negative chemicals that are positive in vitro in mammalian cell assays but are negative in standard in vivo genotoxicity assays or carcinogenicity assays, or induce tumors via an accepted non-genotoxic mode of action e.g., sodium saccharin).

The group also agreed that the conclusion on the preference for a toxicity measure that indicates cell proliferation following the beginning of treatment applies both to in vitro MN assays and to in vitro chromosome aberration assays (where mitotic index is an additional measure, for assays in lymphocytes).

5.1.2. Review of upper limit of cytotoxicity

In the early 1990s expert groups developing the OECD guideline 473 [6], and the IWGTP group [13,14] recommended an upper limit of 50% cytotoxicity for the in vitro chromosome aberration assay. However, the final recommendation in the guideline was ">" 50%, a change that was made for practical purposes to avoid repeated attempts to obtain exactly 50% cytotoxicity. This has unintentionally led to the frequent testing of excessively toxic concentrations. International discussions of a suitable upper limit of toxicity for the in vitro MN assay have led to the recommendation in OECD guideline 487 [9] that the upper limit should be 55 ± 5% toxicity at the top concentration scored. At the current IWGT meeting, it was noted that all 14 chemicals assayed for MN induction in the recent interlaboratory trial were detected without exceeding 50% toxicity by any measure, but some chemicals were positive only at concentrations with toxicity quite close to 50% b [8,22].

5.1.2.1. Conclusions on upper limit of toxicity. The expert working group agreed to accept the cytotoxicity range recommended by OECD guideline 487 [9], namely to achieve 55 ± 5% toxicity at the top concentration scored. This also reinforces the original intent of the guidance for the in vitro chromosome aberration assay, where >50% was intended to target the range close to 50% toxicity.

Conflict of interest

None declared.

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