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 ≥5 days or with 2,4-DAT for ≥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[c]	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[c]	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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