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REVIEW

In vitro genotoxicity testing using the micronucleus assay in cell lines, human lymphocytes and 3D human skin models

Micheline Kirsch-Volders*, Ilse Decordier, Azeddine Elhajouji[1], Gina Plas, Marilyn J. Aardema[2] and Michael Fenech[3]

Laboratorium voor Cellulaire Genetica, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium, [1]Novartis Institutes for Biomedical Research, Translational Sciences, Preclinical Safety, Genetic Toxicology and Safety Pharmacology, CH-4002 Basel, Switzerland, [2]Marilyn Aardema Consulting, LLC, 5315 Oakbrook Dr., Fairfield, OH 45014, USA and [3]CSIRO Food and Nutritional Sciences, Nutritional Genomics, Gate 13, Kintore Avenue, PO Box 10041, Adelaide BC, SA 5000, Australia

*To whom correspondence should be addressed. Tel: +32 2 629 34 23; Fax: +32 2 629 27 59; Email: mkirschv@vub.ac.be

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The toxicological relevance of the micronucleus (MN) test is well defined: it is a multi-target genotoxic endpoint, assessing not only clastogenic and aneugenic events but also some epigenetic effects, which is simple to score, accurate, applicable in different cell types. In addition, it is predictive for cancer, amenable for automation and allows good extrapolation for potential limits of exposure or thresholds and it is easily measured in experimental both *in vitro* and *in vivo* systems. Implementation of *in vitro* micronucleus (IVMN) assays in the battery of tests for hazard and risk assessment of potential mutagens/carcinogens is therefore fully justified. Moreover, the final draft of an OECD guideline became recently available for this test. In this review, we discuss the prerequisites for an acceptable MN assay, including the cell as unit of observation, importance of cell membranes, the requirement of a mitotic or meiotic division and the assessment of cell division in the presence of the test substance. Furthermore, the importance of adequate design of protocols is highlighted and new developments, in particular the *in vitro* 3D human skin models, are discussed. Finally, we address future research perspectives including the possibility of a combined primary 3D human skin and primary human whole blood culture system, and the need for adaptation of the IVMN assays to assess the genotoxic potential of new materials, in particular nanomaterials.

Introduction and toxicological relevance

The presence of micronuclei (MN) in cultured human cells has been reported as early as the 1960s (1) and 1970s (2). The *in vitro* micronucleus test (IVMNT) has evolved into a robust quantitative assay of chromosome damage by the development of the cytokinesis-block technique that eliminated the confounding effects on MN expression by the cytostatic effects caused by poor culture conditions, treatment effects, cell senescence and variability in mitogen response in the lymphocyte test system (3,4). In the cytokinesis-block micronucleus (CBMN) assay, scoring of MN discriminates between once-divided cells that are accumulated and recognised by their binucleated appearance and mononucleated cells that did not divide during the *in vitro* culturing period.

In recent years, the IVMNT has become an attractive tool for genotoxicity testing because of its capacity to detect not only clastogenic and aneugenic events but also some epigenetic effects and its simplicity of scoring, accuracy, wide applicability in different cell types and amenability to automation. More recently, the final draft of its OECD guideline 478 was made available (5). The initial recommendations for this guideline came from two workshops [International Workshops on Genotoxicity Testing, IWGT], which proposed an internationally harmonised protocol designed for both human primary lymphocytes and cell lines (6,7). Validation of the methodology was achieved by the ECVAM (European Centre for the Validation of Alternative Methods) retrospective examination of the existing data published on the IVMNT (8–12) using the modular approach for validation (13). ECVAM confirmed that the IVMNT is reliable, reproducible, transferable and predictive (14) and was endorsed by the ECVAM Scientific Advisory Committee (15,16). The final step before acceptance by the OECD consisted of a interlaboratory exercise to evaluate different measures of cytotoxicity/cytostasis that can be applied when the *in vitro* micronucleus (IVMN) is performed in the absence of cytochalasin B [reviewed in ref. (15)]. The use of the IVMNT within a battery of tests will be defined by the various regulatory bodies responsible for developing such test strategies.

The advantages of the IVMNT, which is discussed in Bonassi *et al.*, Decordier *et al.* and Elhajouji *et al.* (17–19), are well defined: it is a multi-target genotoxic endpoint, predictive for cancer (17); it is amenable for automation (18) and it allows good extrapolation for potential limits of exposure or thresholds (19). In addition, MN can be scored easily measured in a variety of systems, *in vitro* and *in vivo* (20). Implementation of IVMN assays in the battery of tests for hazard and risk assessment of potential mutagens/carcinogens is therefore fully justified.

With this short review, we summarise the major methodologies developed in the past to perform the IVMN assay and describe new developments, in particular the *in vitro* 3D human skin models as an alternative for *in vivo* testing.

Prerequisites for an acceptable IVMN assay

The IVMN assay is a cellular assay where the unit of observation is the cell. Therefore, cell preparation, treatment, fixation, spreading, staining and/or scoring should consider the integrity of the cell membrane. Moreover, since the MN is a small entity independent of the main nucleus, it might easily be lost if inappropriate fixation/spreading procedures are

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applied. Procedures that allow the identification of both cellular and nuclear membranes are needed. Alternative technologies using flow cytometry that require lysis of cell membranes to release and count MN are described by Dertinger *et al.* (21).

In addition to these prerequisites, MN formation resulting from chromosome breakage and/or disturbance of the chromosome segregation machine requires a mitotic or meiotic division and assessment of cell division in the presence of the test substance is compulsory. The cytokinesis-block methodology, based on cytochalasin B inhibition of actin filaments during cytokinesis and the formation of daughter cells (3,4), provided an efficient approach to distinguish between cells that did not divide and those cells that completed nuclear division during *in vitro* culture. Scoring of MN in monucleated cells that are present in the cytokinesis-block assay was shown to be a good indicator for mitotic slippage (22). When using primary cultures (e.g. peripheral blood lymphocytes), it is essential to use the cytokinesis-block method to score MN in once-divided cells only and thus avoid confounding of the observed MN frequency caused by cell division inhibition due to cytotoxicity or poor culture conditions. It is possible to generate false-negative results if MN are not specifically scored in cells that have completed one nuclear division after or during the exposure to the genotoxin (23). In cases when the test compound may interact with actins, the target of cytochalasin B, other alternatives or modifications of the protocol should be recommended (24,25). When using cell lines, the OECD guideline allows the choice between the use of cytochalasin B and the adequate assessment of cell toxicity/cytostasis with cell counts [e.g. relative cell count (RCC), relative increase in cell count (RICC), relative population doubling (RPD)] (26). These parameters were analysed with a selected number of positive controls in an interlaboratory exercise recently published in a special issue of *Mutation Research* (27). It was concluded that all the above measures of cytotoxicity were equally acceptable, but measures of cell proliferation (e.g. RICC, RPD) may help avoid false-positive results. It should be noted that performing the MN assay without cytokinesis-block prevents the possibility of measuring important complementary events such as nucleoplasmic bridge formation, a biomarker of DNA break misrepair or telomere end fusion, which can only be measured in binucleated cells (28).

Comparison of the different methodologies

Tables I–III present an overview of the protocols available for whole blood lymphocyte cultures, isolated lymphocytes and cell lines. The first protocols developed used synchronised cells in order to be able to treat cells in specific cell cycle phases depending on the question of the scientists. The OECD guideline is not restricted to synchronised cells since it aims at maximising the probability of detecting an aneugen or clastogen acting at any stage of the cell cycle; therefore, a sufficient number of cells should be treated with the test substance during all the phases of their cell cycle.

Human lymphocytes

The major advantage of lymphocytes is that they are primary cells, easy to culture in suspension. The choice between whole blood and isolated lymphocytes depends upon the question addressed. Differences in response can be found because erythrocytes in whole blood are the dominant cell type (ratio of ~1 leucocyte: 1000 erythrocytes) and haemoglobin represents an important additional target for reactive molecules that may facilitate metabolic activation or detoxification of the test compound (30,41). The most important differences among the protocols are the hypotonic treatment (critical in particular for image analysis), fixation of the cells (dependent on laboratory preferences) and the final slide preparation. These different parameters significantly influence cell density and cytoplasm preservation. A detailed protocol for isolated lymphocyte and whole blood culture MN assays was recently published and included detailed scoring criteria validated and recommended by the HUMN project consortium (28,42).

Cell lines

Despite some disadvantages at the level of genetic stability, cell lines are often preferred by some laboratories based on ease of handling frozen stocks of cells, lack of variation that can occur in human lymphocyte donors and existence of large historical databases. The OECD guideline 478 for the IVMN refers to the extensive data supporting the validity of the assay using various rodent cell lines (CHO, V79, CHL and L5178Y). These include, in particular, the international validation studies co-ordinated by the Société Française de Toxicologie Génétique (8–12) and the reports of the IWGT (4,17) as reviewed in the ECVAM retrospective validation (14).

For review of the standard methodologies, see Table III. In a recent paper (43), the results of IVMNT across 12 different laboratories for 14 different chemicals in five different cell lines were summarised. It was demonstrated that cytotoxic and cytostatic measures performed in L5178Y, TK6, CHO, CHL and V79 in the absence of cytochalasin B at or below target range toxicity ($55 \pm 5\%$) were adequate to perform the MN test when calculated as RCC, RICC or RPD. The use of the human TK6 lymphoblastoid cell line (44) and the human HepG2 cells (45,46) and Syrian hamster embryo cells (47) has been described, although they have not been used in validation studies.

Although all these validation studies indicate that the use of cell lines can be recommended, it should be underlined that most of the cell lines are deficient in p53 or apoptosis-controlling genes that lead to higher frequencies of MN (48) and is thought to lead to positive results that are not confirmed *in vivo* in some cases. In contrast, for mechanistic studies, the use of cell lines can be very useful. As examples, metabolically competent hepatoma cell lines, such as HepG2 and Hep3B (36,46,49), and rodent hepatoma cell lines (50) were studied. It was shown that among the human hepatoma cell lines, HepG2 was more sensitive than Hep3B and that variation between clones of HepG2 exists. The fact that HepG2 is more sensitive towards some genotoxins and enables detection of genotoxic carcinogens which gives negative results in other currently used bioassays suggests that in some cases, they might be more suitable than cell lines currently used for routine screening [reviewed in refs (46,49)]. Other human cell lines, such as HepaRG, stable cell lines from organs other than the liver, primary rat hepatocytes [(51) and human stem cells (the target for carcinogenicity] have not been used for genotoxicity investigations and should be considered for evaluation. Genetic engineering is also a valuable tool to incorporate missing enzyme systems into target cells [for review see Kirkland (52)]. A collaborative research programme is needed to identify,

Table I. Overview of the protocols for MN assay in whole blood lymphocyte cultures

	Van Hummelen and Kirsch-Volders (29)[a]	Elhajouji *et al.* (30)[b]	Decordier *et al.* (18)[c]	Draft OECD guideline 487 MNvit (5)[d]	
Time: 0	Culture set-up in medium, supplemented with calf serum and PHA				
Time: 24 h	Treatment				
Time: 44 h	Addition of cytochalasin B in a final concentration of 6 μg/ml	Addition of cytochalasin B in a final concentration of 3–6 μg/ml	Addition of cytochalasin B in a final concentration of 6 μg/ml	Treatment (short exposure 3–6 h), with and without S9	If short treatment is negative or equivocal: treatment (long exposure, without S9) + cytochalasin B
Time: 47–50 h				Removal of treatment medium, addition of fresh medium + cytochalasin B	
Time: 72 h	2 washing steps (RPMI + 2% FCS) and a mild hypotonic treatment (RPMI/H_2O 4:1), followed by smearing and fixation in M/A 3:1	Mild centrifugation—mild hypotonic treatment with cold 75 mM KCl, followed by 3 fixation steps in M/A 3:1, supplemented with formaldehyde at 1st fixation. Spotting onto slides.	Mild centrifugation—mild hypotonic treatment (15 min 110 or 90 mM KCl at 4°C), followed by 3 fixation steps in M/A 3:1, supplemented with formaldehyde at 1st fixation. Spotting onto slides.		
Time: 80–92 h				Harvesting, with or without hypotonic treatment	
	Staining: Giemsa 5% in Sörensen buffer, filtered once	Staining: Giemsa or fluorescent DNA-specific dyes	Staining: Giemsa 5% in Sörensen buffer, filtered twice	Staining: Giemsa or fluorescent DNA-specific dyes	
Advantages	Safe hypotonic treatment, little chance of cytoplasm loss. Very bright staining. Good contrast between cytoplasm and nuclear material.	No loss of cells due to mechanical damage. Image suitable for automation.	Adding an extra cold step improves reproducibility and allows for automation with IMSTAR Pathfinder.		
Disadvantages	Mechanical damage to cells towards the end of the feather, only top half of the slide can be used for scoring. Thickness of smear difficult to standardise. Not suitable for automation.	Subtle balance between optimal swelling and loss of cytoplasm. Not always reproducible. With Giemsa, less contrast between cytoplasm and nuclear material.	Narrow margins for optimal cell detection. Giemsa impurities or other artefacts can seriously impede automatic detection.		

[a]Modified from Högstedt (31), Fenech and Morley (3), Maki-Paakkanen and Norppa (32) and Thomson and Perry (33).
[b]Modified from Tates *et al.* (34), Surrales *et al.* (35) and Darroudi *et al.* (36).
[c]Modified from Elhajouji *et al.* (30).
[d]Modified from Kirsch-Volders *et al.* (6’7’37).

further develop and evaluate new cell systems with appropriate sensitivity but improved specificity.

Importance of adequate protocol design

The design of the protocol is crucial in the generation of accurate results and assessment of the genotoxic potential of the test substance. The choice of the cellular system, treatment duration, the use of a cytokinesis blocker, the class of the test compound or the addition of metabolic components may significantly influence the test outcome.

Either using primary cells or established cell lines, cell cycle duration is essential in defining culture duration. The OECD draft guideline 478 stresses that cell cycle duration of the cell system should be known and should guide the treatment and culture duration. The treatment duration should cover all cell cycle phases to allow a proper interaction of the test substance with all potentially relevant cellular components for gentoxicity. In general, the cells are treated at an initial phase with the test substance for a short period (3–6 h) considered sufficient to detect the majority of clastogens and aneugens followed by a recovery phase (8). The cell harvesting is done at 1.5–2.0 normal cell cycle (4). The use of metabolically competent cells (engineered cells) or the addition of S9 metabolic activation system is required for the detection of genotoxins requiring metabolic activation. If negative or equivocal results are obtained, an extended treatment covering 1.5–2.0 duration of the cell cycle is performed. If the mode of action of the test substance is known to significantly interfere with the cell cycle (e.g. nucleoside analogues), treatment and recovery durations are adapted. Finally, the tested concentrations should be closely spaced to cover concentrations with little/no cytotoxicity to clearly cytotoxic concentrations up to 55 ± 5% cytotoxicity (5,51). If the test substance did not exhibit significant cytotoxicity or precipitation at the highest dose, selection should be limited to 0.01 M, 5 mg/ml or 5 μl/ml, whichever is the lowest (5). It is important to note that discussions on lowering the 0.01 M limit were part of a recent IWGT meeting

Table II. Overview of the protocols for MN assay in isolated lymphocyte cultures

	Van Hummelen *et al.* (38)[a]	Elhajouji *et al.* (39)	Decordier *et al.* (18) [b]	Draft OECD guideline 487 MNvit (5)[c]	
Time: 0	Isolation of lymphocytes on density gradient, set-up at 0.5–1.0 10^6 cells/ml of culture medium				
Time: 24 h	Treatment				
Time: 44 h	Addition of cytochalasin B in a final concentration of 6 μg/ml			Treatment (short exposure 3–6 h), with and without S9	If short treatment is negative or equivocal: treatment (long exposure, without S9) + cytochalasin B
Time: 47–50 h				Removal of treatment medium, addition of fresh medium + cytochalasin B	
Time: 72 h	Cytospin (700 rpm, 5 min), fix 100% methanol	Mild centrifugation—mild hypotonic treatment with cold 75 mM KCl, followed by 3 fixation steps in M/A 3:1. Spotting onto slides.	Mild hypotonic treatment (15 min 110 or 90 mM KCl at 4°C), followed by 2 fixation steps in M/A 3:1, supplemented with formaldehyde at 1st fixation. Spotting onto slides.		
Time: 80–92 h				Harvesting, with or without hypotonic treatment	
	Staining: Giemsa 5% in Sörensen buffer, filtered once	Staining: Giemsa or fluorescent DNA specific dyes	Staining: Giemsa 5% in Sörensen buffer, filtered twice	Staining: Giemsa or fluorescent DNA-specific dyes	
Advantages	Very easy and fast fixation—no need for hypotonic treatment. Good contrast between cytoplasm and nuclear material. Many cells on a small surface allow fast scoring.	Individual cells separated from each other allow FISH interpretation and automation.	Adding an extra cold step improves reproducibility and allows for automation with IMSTAR Pathfinder. Decreasing the number of fixation steps reduces cell loss.		
Disadvantages	Difficult interpretation if cells are too dense. Not suitable for FISH interpretation or automation.	Subtle balance between optimal swelling and loss of cytoplasm. Not always reproducible.	Narrow margins for optimal cell detection. Giemsa impurities or other artefacts can seriously impede automatic detection.		

[a]Modified from Vian *et al.* (40).
[b]Modified from Elhajouji *et al.* (39).
[c]Modified from Kirsch-Volders *et al.* (6'7'37).

(Galloway, S., Lorge, E., Aardema, M. J., Eastmond, D., Fellows, M., Heflich, R., Kirkland, D., Levy, D. D., Lynch, A., Marzin, D., Morita, T., Schuler, M., Speit, G., in preparation).

New developments: 3D human reconstructed skin MN assays

A novel *in vitro* human reconstructed skin micronucleus (RSMN) assay that measures MN induced in dividing basal cell keratinocytes of the EpiDerm™ 3D human skin model has recently been developed (53,54). The RSMN assay was designed to help address the challenges created by the March 2009 ban on *in vivo* genotoxicity testing of cosmetics ingredients for products marketed in Europe [EU 7th Amendment to the Cosmetics Directive (55)] and large-scale chemical evaluation programmes such as REACh (Registration, Evaluation, Authorisation and Restriction of Chemical substances) (56) for which the conduct of *in vivo* assays on tens of thousands of chemicals is impractical. Expert opinions indicate that the inability to conduct *in vivo* follow-up testing for cosmetics will make the evaluation of the genotoxicity of these ingredients impossible in many cases (57,58). Moreover, the issue of false-positive results in the standard *in vitro* genotoxicity assays and challenges with follow-up testing are recognized as a critical issue in the global genetic toxicology community. One approach to address this issue is to develop improved *in vitro* genotoxicity assays. One focus has been on the use of 3D human reconstructed skin (RS) models for development of a new more physiologically relevant and predictive *in vitro* MN assay. We selected the EpiDerm™ model (MatTek Corporation; www.mattek.com) constructed from primary neonatal epidermal foreskin-derived keratinocytes. The model consists of a multilayered differentiated tissue containing the dividing basal cell layer along with spinous, granular and cornified layers resembling the normal human epidermis. Since this model has a functional stratum corneum, it provides a way to achieve a more relevant exposure to the dividing basal cells that are the target cells for MN formation. This allows realistic concentrations of chemicals and drugs to be tested at the skin surface and metabolic effects that might occur as the tested agent that diffuses from the model skin surface to the basal layer.

RS models prepared from primary cells are expected to have more normal DNA repair and cell cycle control than transformed cell lines and human metabolic capability that is more relevant for dermally applied chemicals than the

Table III. Overview of the protocols for MN assay in cell lines

Modified from Kirsch-Volders *et al.* (7)					Draft OECD guideline 487 MNvit (5)
Time: 0	Culture set-up in medium dependent on the type of cell line used				
Time: 24 h	Treatment				
Time: during first cell cycle following treatment (time dep. on type of cell line used)	Addition of cytochalasin B (conc. dep. on the type of cell line used)		Without addition of cytochalasin B		Same protocol with and without cytochalasin B
Time: prior to the second mitosis (time dep. on type of cell line used)	Harvesting of adherent cell line	Harvesting of cell lines in suspension	Time:- Short treatment with or without S9: at 27–30 h: removal of the test compound and removal of treatment medium, addition of fresh medium, harvesting after 2 cell cycles after start of treatment -Continuous treatment without S9Harvesting after 2.5 cell cycles after start of treatment		Time: - After 27–30 h: removal of the test compound (short treatment with or without S9) and removal of treatment medium, addition of fresh medium, harvesting after 1.5–2 cell cycles after start of treatment -After 1.5–2 cell cycles in presence of cytochalasin B, harvest at the end of the exposure period after start of treatment (continuous treatment without S9) or -After 1.5–2 cell cycles removal of the test compound, addition of fresh medium and cytochalasin B, harvest 1.5–2 cell cycles later
	-Trypsinisation, cytospin, fix in 100% methanol -Trypsinisation, cold hypotonic shock (75 mM) or 1% sodium citrate, fixation in E/A 3:1 or M/A 3:1, suspended in 1% M with 1% A, spotting onto slides -Cells grown onto slides/coverslips, fixed in 100 methanol	-Cytospin, fix in 100% methanol -Cold hypotonic shock (75 mM), fixation in M/A 3:1, A, spotting onto slides	-Trypsinisation, cytospin, fix in 100% methanol -Trypsinisation, cold hypotonic shock (75 mM) or 1% sodium citrate, fixation in E/A 3:1 or M/A 3:1, suspended in 1% M with 1% A, spotting onto slides -Cells grown onto slides/coverslips, fixed in 100 methanol	-Cytospin, fix in 100% methanol -Cold hypotonic shock (75 mM), fixation in M/A 3:1, A, spotting onto slides	Idem as for methods modified from Kirsch-Volders *et al.* (7)
	Staining with Giemsa or fluorescent DNA specific dyes				

exogenous rodent metabolising enzymes S9 mix currently added in standard *in vitro* genotoxicity assays. As recently reviewed (59,60), most drug metabolising enzymes including Phase 1 and Phase II pathways are present in skin, more specifically in the epidermis, with higher amounts occurring in the keratinocytes compared to other cell types. Despite the fact that most metabolising enzymes are present in skin, the activity is typically lower than in liver though the activity of skin is still considered relevant from a toxicological standpoint due to localised metabolism that can occur. Recent studies from our laboratory demonstrated that aromatic amine hair dye constituents *p*-aminophenol and *p*-phenylenediamine are *N*-acetylated (a detoxification step) in the EpiDermTM model consistent with what has been shown in normal human scalp (61). Using microarray analysis, expression of 139 xenobiotic metabolism genes in the EpiDerm™ model were compared to biopsied human skin. The results demonstrated that expression of xenobiotic metabolism genes in the EpiDerm™ model is very similar to that in human skin (64). Overall, the expression of Phase II enzymes appeared to be more pronounced compared to Phase I enzymes both in the EpiDerm™ model and human skin, consistent with a role of skin in detoxification of xenobiotics.

Flamand *et al.* (65) described another promising related methodology that measures MN in L5178Y cells co-cultured in the EpiSkin® model. In this same paper, another endpoint, the Comet assay, in the EpiSkin® model is also described (65).

The protocols for MN assessment in the 3D skin models are quite similar to the OECD guideline for *in vitro* MN assays; descriptions of the RSMN assay and parameters for a valid test have been published (53,54,66). Some validation exercises have started. The RSMN assay in EpiDerm™ was compared across three laboratories using model genotoxins and four dermal non-carcinogens, previously reported to be genotoxic in standard assays; the latter being negative in RSMN indicating that the

assay has promise as an assay with higher predictivity than standard *in vitro* genotoxicity assays (66). Importantly, there was good reproducibility between different EpiDerm™ 3D models containing keratinocytes isolated from human foreskin from four different donors. This is important since these primary cells have a finite lifespan and must be replaced by a new donor when batches of cells are depleted. A larger number of comparisons are needed to verify the degree of inter-individual variation that may be expected in human populations depending on their genetic and nutritional status.

Recently, the RSMN assay was expanded to other laboratories as part of a prevalidations project sponsored by The European Cosmetics Association (COLIPA), with contribution from the ECVAM. Since the EpiDerm™ models are manufactured in the USA, it was important to establish whether they could be shipped to Europe and used successfully in the RSMN assay. Intra- and interlaboratory reproducibility of the RSMN assay was established by testing three coded chemicals: *N*-ethyl-*N*-nitrosourea, cyclohexanone and mitomycin C (67). All chemicals were correctly identified by the laboratories. These results support the conclusion that the RSMN in the EpiDerm™ model appears to be a relevant *in vitro* system for the study of cutaneous exposures, metabolism and toxicity including genotoxicity. At this point in time, the RSMN assay would be useful as a follow-up test for dermally applied chemicals that are positive in current *in vitro* genotoxicity tests as discussed for cosmetics in Pfuhler *et al.* (68). If the performance of the assay is demonstrated to be better than current *in vitro* cytogenetic assays (i.e. similar high sensitivity but greater specificity than the *in vitro* MN or *in vitro* chromosome aberration assays), it is envisaged that the RSMN assay could even be a replacement for these tests in Tier I batteries in the future.

Summary of main achievements in this area of research

The standard IVMN assay went through a long evolution from the research bench to a finalised OECD guideline. It is already applied in many laboratories and provides a well-validated tool to assess hazard (and risk) of genotoxicants. The main achievements over the past three decades with regards to the IVMN assay are as follows.

(i) The recognition that false-negative results or an underestimate of MN induction could be obtained if scoring of MN was not discriminating between once-divided cells and non-divided cells following exposure to the test chemical or physical agent.
(ii) The development of the cytokinesis-block block method as a robust technique for identifying once-divided cells unequivocally by their binucleated appearance.
(iii) The cytokinesis-block MN assay's evolution into a comprehensive cytome technique that apart from MN also enabled robust quantification of other highly relevant and related biomarkers of chromosome instability such as nucleoplasmic bridges and nuclear buds measured in binucleated cells, the measurement of nuclear division rate as well as two modes of cell death (necrosis and apoptosis) (28).
(iv) The validation of use of molecular probes to interrogate the mechanism of MN formation (e.g. centromere-positive or centromere-negative MN for identifying chromosome loss or chromosome breakage events, respectively) as well as malsegregation of specific chromosomes if chromosome-specific centromere probes are used (39,69).
(v) Furthermore, the relevance and validation of the cytokinesis-block MN assay has been strongly enhanced by prospective epidemiological studies showing that the MN biomarker in binucleated lymphocytes within the CBMN cytome assay predicts the risk for pregnancy complications, cardiovascular disease mortality and cancer (70–74).
(vi) These achievements are being extended by the wide-spread adoption of the *in vitro* primary lymphocyte MN assay using the cytokinesis-block technique for both *in vitro* genetic toxicology testing and *in vivo* biomonitoring. This now enables an almost completely harmonised approach of evaluation of human genotoxic risk assessment because the same system can be used not only for *in vitro* testing but also as a measure of *in vivo*-induced DNA damage (7).
(vii) Assessment of MN in new models such as RS. The RSMN assay serves as an important example of a paradigm shift in the field of genetic toxicology testing away from the classical *in vitro* assays with standard cells/cell lines, to use of 3D human tissue models to achieve more relevant testing strategies.

Knowledge gaps and future research

At the present time, one of the major knowledge gaps in using the IVMN assay is the adaptation for the assessment of nanomaterials. The questions relate to an adequate protocol design in order to allow optimal uptake of the insoluble nanomaterials and their presence within the cells during mitosis. Proposals are formulated by Gonzalez *et al.* (25).

The use of nucleoplasmic bridges in combination with MN provides important mechanistic information and added capacity to detect dicentric chromosomes that may arise due to misrepair of DNA breaks, telomere end fusions or inhibition of chromatid separation during anaphase. Studies are needed to test whether including measurement of nucleoplasmic bridges provides better sensitivity and specificity of the INMNT to detect genotoxins and carcinogens (for review see ref. 75).

As far as the 3D human skin model is concerned, further work is needed to:

(i) obtain data on a wide domain of chemicals including chemicals that are genotoxic in a variety of *in vivo* in tissues including skin, bone marrow, blood, liver, etc.,
(ii) investigate the functional metabolic activity of the RS system and develop an appropriate protocol for chemicals requiring metabolism,
(iii) automation of the analysis in the RSMN assay to expedite generation of data,
(iv) establish the assay in other commercially available RS models,
(v) investigate RSMN assay in models consisting of different cell types including full thickness models, combining primary human whole blood cultured below the skin for additional MN analysis,
(vi) development of *in vitro* MN assays in other 3D reconstructed human models to address questions related to genotoxicity in specific tissues like oral mucosa, tracheal/bronchial, liver, etc. and
(vii) develop assays for measurement of other endpoints to compliment MN in RS models, such as the Comet assay, the subject of work in the ongoing COLIPA sponsored project.

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