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Mutation Research/Reviews in Mutation Research, 2014; 759:49-58

Originally published at:
http://doi.org/10.1016/j.mrrev.2013.12.001

Mutation Research 759 (2014) 49–58

Contents lists available at ScienceDirect

Mutation Research/Reviews in Mutation Research

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

Commentary

Commentary: Critical questions, misconceptions and a road map for improving the use of the lymphocyte cytokinesis-block micronucleus assay for *in vivo* biomonitoring of human exposure to genotoxic chemicals—A HUMN project perspective☆

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ARTICLE INFO

Article history:
Received 10 October 2013
Received in revised form 24 December 2013
Accepted 26 December 2013
Available online 8 January 2014

Keywords:
Micronucleus
Cytokinesis-block
Lymphocytes
Chemical genotoxins
Biomonitoring
In vivo

ABSTRACT

The lymphocyte cytokinesis-block micronucleus (CBMN) assay has been applied in hundreds of *in vivo* biomonitoring studies of humans exposed to genotoxic chemicals because it allows the measurement of both structural and numerical chromosome aberrations. The CBMN cytome assay version which, apart from measuring micronuclei (MN) already present in cells *in vivo* or expressed *ex vivo*, also includes measurement of nucleoplasmic bridges (NPB), nuclear buds (NBUD), necrosis and apoptosis, is also increasingly being used in such studies. Because of the numerous published studies there is now a need to re-evaluate the use of MN and other biomarkers within the lymphocyte CBMN cytome assay as quantitative indicators of exposure to chemical genotoxins and the genetic hazard this may cause. This review has identified some important misconceptions as well as knowledge gaps that need to be addressed to make further progress in the proper application of this promising technique and enable its full potential to be realised. The HUMN project consortium recommends a three pronged approach to further improve the knowledge base and application of the lymphocyte CBMN cytome assay to measure DNA damage in humans exposed to chemical genotoxins: (i) a series of systematic reviews, one for each class of chemical genotoxins, of studies which have investigated the association of *in vivo* exposure in humans with MN, NPB and NBUD induction in lymphocytes; (ii) a comprehensive analysis of the literature to obtain new insights on the potential mechanisms by which different classes of chemicals may induce MN, NPB and NBUD *in vitro* and *in vivo* and (iii) investigation of the potential advantages of using the lymphocyte CBMN cytome assay in conjunction with other promising complementary DNA damage diagnostics to obtain an even more complete assessment of the DNA damage profile induced by *in vivo* exposure to chemical genotoxins in humans.

1. Introduction

The cytokinesis-block micronucleus (CBMN) assay in human lymphocytes has become one of the most widely used methods for measuring structural and numerical chromosomal changes in human cells *in vitro* and *in vivo* [1–4]. Micronuclei (MN) result from acentric chromosome fragments or whole chromosomes lagging behind during metaphase/anaphase transition, and require cell division *in vivo* or *in vitro* to be expressed [1–3]. The use of the CBMN assay in *in vitro* genetic toxicology testing is well established and in fact it has become an accepted standard method to assess the genotoxic hazard of chemicals which led to the development of an OECD (Organisation for Economic Cooperation and Development) guideline for this purpose [2]. For *in vitro* testing cultures of unsynchronised mitogen-stimulated lymphocytes are treated *in*

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1383-5742
http://dx.doi.org/10.1016/j.mrrev.2013.12.001

vitro with a single compound and fixed after completing the following mitosis to measure micronuclei in once-divided cells, thus maximising the efficiency of detecting aneugens or clastogens acting at different stages of the cell cycle [1–3]. Parallel micronucleus scoring in non-divided cells, reflecting slower cycling cells and/or mitotic slippage, is also recommended in particular if an aneugenic mode of action is suspected [2,3]. The CBMN assay is also widely used in human biomonitoring of *in vivo* exposure to genotoxins and has become a standard biodosimetry method endorsed by the International Atomic Energy Agency and the World Health Organization for measuring exposure to ionising radiation [4]. The assay measures micronuclei (MN) and other nuclear anomalies in *ex vivo* mitogen stimulated lymphocytes from *in vivo* exposed persons, integrating in this way *in vivo* systemic exposure of lymphocytes and *in vivo/ex vivo* response to the genotoxic stress. Its predictivity for the detection of genetic risks is supported by the fact that it allows measurement at the single cell level of both structural and numerical chromosome aberrations [1,3]. Moreover, its predictivity for cancer was demonstrated in humans [5]. However, the use of the CBMN assay for detecting *in vivo* exposure to genotoxic chemicals is somewhat controversial because of the extremely wide diversity of chemicals, the multitude of direct or indirect mechanisms of their interaction with the genome, the wide spectrum of DNA lesions they may induce and the variety of cellular death/survival responses they may trigger. Comprehensive reviews about the mechanistic, methodological and epidemiological aspects of the use of micronucleus assays were recently published [1–7], but systematic reviews on the application of the lymphocyte CBMN assay for *in vivo* biomonitoring of chemical exposure are limited to only a few categories of chemicals such as pesticides [8].

2. Key questions regarding the use of the lymphocyte CBMN assay for *in vivo* biomonitoring of human exposure to genotoxic chemicals

Despite the extensive evidence available in the literature, a number of key aspects remain unresolved about the origin of MN in the lymphocyte CBMN assay following *in vivo* genotoxic chemical exposure and the significance of MN as indicators of chemical carcinogenesis. These issues raise important questions that deserve further investigation:

1. What are the direct or indirect mechanisms by which different classes of genotoxic chemicals induce structural and/or numerical chromosome aberrations and which of these lead to MN formation?
2. Is the CBMN assay as conventionally used in human biomonitoring able (or sensitive enough) to detect exposure to all classes of chemical genotoxins?
3. Which classes of chemical genotoxins can be detected and which ones cannot be detected by the lymphocyte CBMN assay?
4. Can the conventional lymphocyte CBMN assay also be used to measure *in vivo* induced MN that are already present in the cells?
5. Can the CBMN assay protocol be modified to improve the sensitivity to DNA lesions that are not efficiently converted to micronuclei?
6. Does the inclusion of other nuclear anomalies such as nucleoplasmic bridges and nuclear buds in the "cytome" version of the method improve the sensitivity of the CBMN assay for detecting *in vivo* chemical genotoxin exposure?
7. Which other complementary DNA damage biomarkers should be used in combination with the CBMN cytome assay to enhance the detection of DNA damage induced by chemical genotoxins?

Considerations and some answers related to these questions are addressed below.

2.1. What are the direct or indirect mechanisms by which different classes of genotoxic chemicals induce structural and/or numerical chromosome aberrations and which of these lead to MN formation?

Direct mechanisms by which chemical genotoxins can cause structural chromosome aberrations or numerical chromosome changes mainly include the formation of small or bulky adducts, DNA strand cross-links, DNA-protein cross-links and DNA strand breaks [9,10]. Indirect mechanisms include inhibition of DNA repair, impairment of chromosome segregation, disruption of mitotic checkpoints machinery, inhibition of apoptosis, perturbation of cytokinesis, inhibition of enzymes involved in the maintenance of DNA methylation, and induction of inflammation and/or mitochondrial dysfunction leading to increased oxidative stress [3,11].

MN arise mainly from either acentric chromosome fragments or whole chromosomes. Acentric chromosome fragments originate from unrepaired DNA strand breaks or misrepair of DNA strand breaks leading to the formation of an acentric chromosome fragment which often occurs in conjunction with formation of a dicentric chromosome [3,4,11]. MN may also arise when a dicentric chromosome breaks in more than one site when stretched to the opposite poles of a cell during anaphase [3,11]. Malsegregation of whole chromosomes may be induced if the chemical either causes centromere or kinetochore malfunction or disrupts the mitotic spindle or centrosome [3,6,11]. The direct mechanism of DNA damage induction has been demonstrated for only a limited number of genotoxic chemicals while for most of the thousands of man-made or natural chemicals neither the direct or indirect mechanism of DNA damage induction has been established [9,10]. Furthermore interactive effects with endogenous genotoxins in the body fluid exposome (*e.g.* reactive oxygen or nitrogen species, acetaldehyde, high free iron concentration *etc.*) is virtually unexplored.

The known mechanisms of how chemicals may induce MN formation are presented schematically in Fig. 1, although there are likely to be several others that we are not yet aware of. The various types of chromatid and chromosome aberrations that contribute to MN and NPB formation are illustrated in Fig. 2.

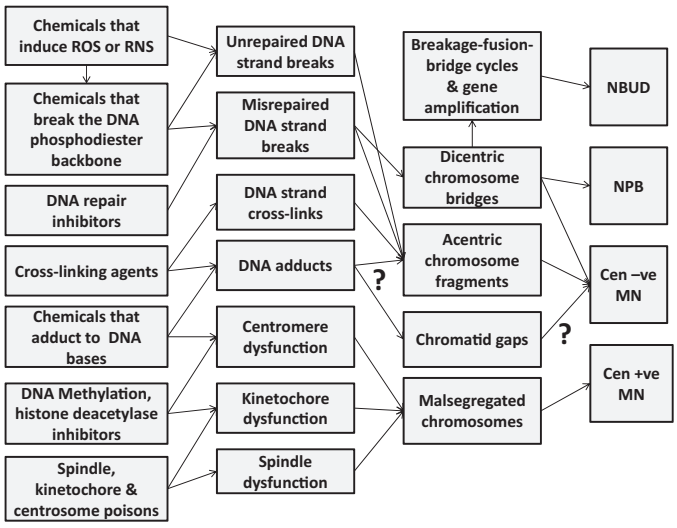

Fig. 1. The known mechanisms by which genotoxic chemicals may induce micronuclei and nucleoplasmic bridges in cytokinesis-blocked lymphocytes. Cen−ve, centromere negative; Cen+ve, centromere positive; NBUD, nuclear buds; NPB, nucleoplasmic bridges; MN, micronuclei; ROS, reactive oxygen species; RNS, reactive nitrogen species. The question mark (?) indicates insufficient knowledge about the mechanism and/or some uncertainty about the connection between the two related events.

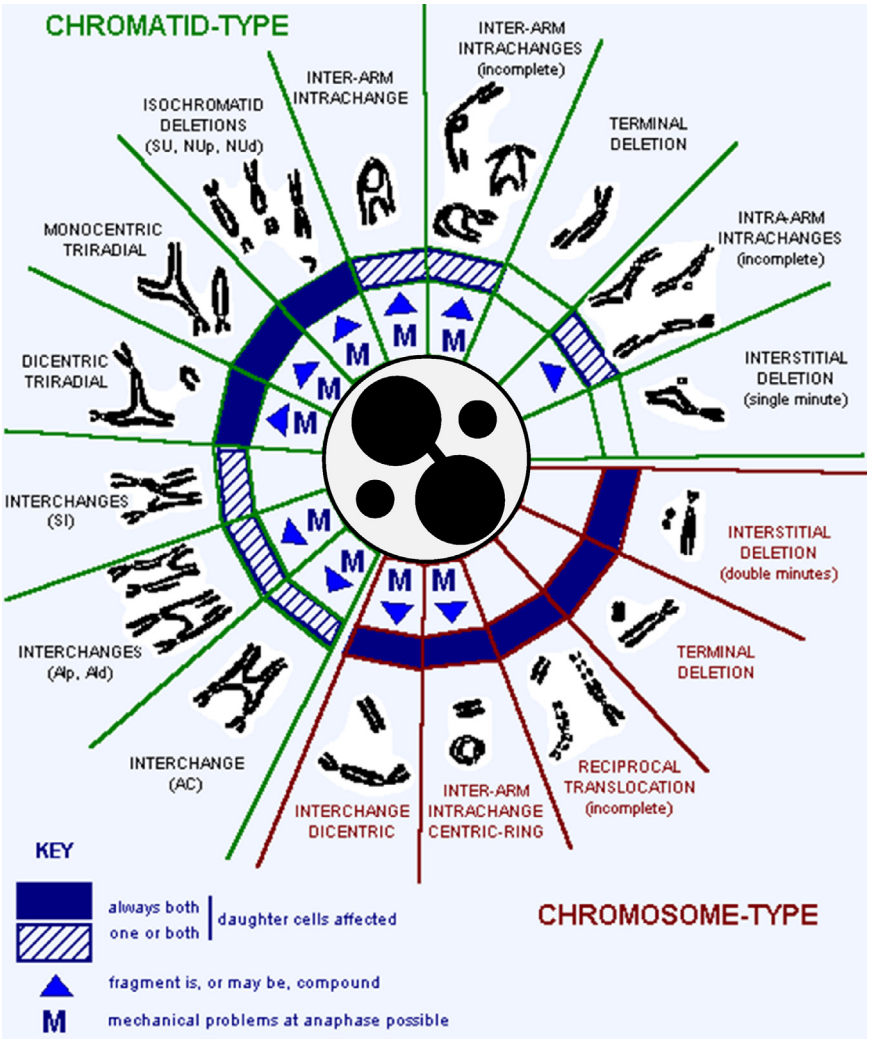

Fig. 2. The principal chromatid and chromosome aberrations that contribute to MN and nucleoplasmic bridge formation in the CBMN cytome assay.
Adapted from Savage JRK (2000) Micronuclei: pitfalls and problems. Atlas Genet Cytogenet Oncol Haematol (http://atlasgeneticsoncology.org/Deep/MicronucleiID20016.html).

2.2. Is the lymphocyte CBMN assay as conventionally used in human biomonitoring able (or sensitive enough) to detect exposure to chemical genotoxins?

The conventional use of the CBMN assay in human biomonitoring of chemical genotoxin exposure involves the collection of a blood sample following acute or chronic *in vivo* exposure to the suspected chemical agent or complex mixture. The lymphocytes in the blood sample are then stimulated to divide *ex vivo* using a mitogen, and MN scored in cells that have divided once which are recognised by their binucleated appearance after cytokinesis-block (using cytochalasin-B) of the cells from the first mitotic cycle [1–3,6]. The bulk of peripheral blood lymphocytes are long-lived T cells and it is these cells that are mainly stimulated to divide by the mitogen used in most studies (*i.e.* phytohaemagglutinin). A proportion of these lymphocytes may already contain MN induced *in vivo* and many of them may have accumulated various types of DNA damage depending on the "exposome", *i.e.* the exposure to the wide range of genotoxins in body fluids resulting from cellular metabolism including inflammation, and/or from environmental genotoxins absorbed into the body that may be either metabolically activated or detoxified [12,13]. Therefore, it is ultimately the entire exposome resulting from systemic exposure that determines the profile of genotoxic insult that the lymphocytes experienced before being stimulated to divide *ex vivo* to express MN in the CBMN assay. It is the nature of the *in vivo* exposome in combination with the individual's genetic and epigenetic background that ultimately determines the expression of MN *ex vivo* after one nuclear division.

Specific *in vitro* experiments with the CBMN assay in which lymphocytes were exposed only in G0 phase (*i.e.* prior to mitogen stimulation) show that the MN index in the CBMN assay can efficiently detect exposure to agents that predominantly induce DNA strand breaks because these lead to acentric chromosome fragments within one cell cycle if they are left unrepaired or if they are mis-repaired [3,14]. Repair of double DNA strand breaks can occur during G0, G1 and G2 with either the error prone non-homologous end-joining (NHEJ) or the error free homologous recombinational repair (HRR) mechanism [15] while repair of single strand breaks occurs *via* a global pathway involving PARP1 as the sensor and XRCC1 as the molecular scaffold protein [16]. Genetic defects in either of these pathways can lead to acentric fragment and dicentric chromosome formation. Acentric chromosome fragments lead to MN formation because the fragments cannot engage with the spindle and thus are not included in the main nuclei during anaphase/telophase.

Some chemicals may predominantly, but not necessarily exclusively, induce either small or bulky DNA adducts. The

presence of DNA adducts normally triggers the excision repair pathways that lead to the excision of the damaged base or template, producing temporary abasic sites, gap-filling by DNA polymerase and ligation of the newly synthesised DNA strand to the adjacent DNA [17]. Failure of any of these processes can lead to the accumulation of residual unrepaired DNA adducts, abasic sites and single-stranded DNA breaks. These DNA lesions may, to varying extents, depending on the adduct and the repair capacity of the cells, lead to replication stress during S-phase and the formation of single or double-stranded DNA breaks [18,19]. Chemicals that predominantly induce DNA adducts that cause stalling of DNA replication forks appear to induce mainly chromatid-type aberrations such as chromatid breaks, chromatid gaps and chromatid rearrangements involving only one chromatid of the chromosome pair at metaphase [20–22] (Fig. 2). The efficiency with which these chromatid-type aberrations are converted to MN or other nuclear aberrations is unknown. The limited evidence available appears to suggest that some chemicals (*e.g.* methylnitrosourea) or physical agents (ultraviolet radiation) that mainly induce DNA base adducts or lesions may not efficiently induce MN if lymphocytes are exposed in the G0 phase only [23]. This relative insensitivity may be overcome by using a modified CBMN assay protocol in which cells are treated with cytosine arabinoside (ARA-C) during G1 prior to S-phase to inhibit the gap-filling step during base excision repair and thus converting base excision-repair sites into abasic sites which leads to single or double strand break formation (if the excision sites are in close proximity across strands) in G1 phase [23,24] and ultimately converted to chromatid aberrations that can be expressed as MN.

The lymphocyte CBMN assay is also sensitive *in vitro* and *ex vivo* to DNA inter-strand cross-linking agents such as acetaldehyde and mitomycin-C as well as DNA-protein cross-linking agents such as formaldehyde [25–30]. The Fanconi anaemia/BRCA pathway is mainly involved in the repair of these important DNA lesions [31,32]. Acetaldehyde and formaldehyde are generated during normal metabolism and are also amongst the most ubiquitous man-made environmental genotoxins/carcinogens [25,28,30,31]. The precise mechanisms by which these agents cause MN is not known in detail but they could cause chromosome- and chromatid-type aberrations in lymphocytes possibly due to indirect induction of DNA strand breaks as a result of incomplete DNA repair of the cross-links and the consequent replication stress events from stalling replication forks [18–32].

Other classes of chemicals that can result in MN formation are those affecting the structure of critical regions of the chromosomes (*e.g.* centromeres, kinetochores, cohesins, telosome proteins) and the mitotic or cytokinetic checkpoints machinery (*e.g.* centrosomes, microtubules) required for accurate segregation of chromosomes during mitosis. Some chemical agents may affect chromosomal instability indirectly. For example, occupational exposure to nitrous oxide (an anaesthetic gas used routinely in hospitals), causes inhibition of methionine synthase by oxidising and inactivating its cofactor vitamin B12 leading to a reduction in methylation capacity of cells and induction of MN *in vivo* [33,34]. Hypomethylation of centromeric DNA leads to despiralisation of the centromere and its dysfunction leading to chromosome malsegregation and MN formation [35,36]. Some of the most potent MN inducers are DNA methyltransferase inhibitors such as 5-azacytidine and mutations in DNA methyltransferase genes [37,38].

The mitotic process involves a complex choreography of multiple proteins and checkpoints and it is, therefore, not improbable that environmental chemicals which can adduct to mitotic proteins may also have significant impacts on this process *in vivo*. Given that a large proportion of spontaneous MN are centromere positive it is reasonable to be concerned about our lack of knowledge of the environmental causes and consequences of this important genetic pathology. Although adducts to proteins in the mitotic machinery may indicate risk of mitotic defects, chromosome malsegregation is currently best analysed directly by the CBMN assay in combination with centromere specific probes to identify MN originating from whole chromosomes or abnormal chromosome distribution amongst nuclei in a binucleated cell [6,11]. Whether the *in vivo* exposure to such agents causes MN formation *ex vivo* in the CBMN assay was almost never assessed and can only be expected to detect these exposures if the *in vivo* exposure causes long lasting damages to the relevant chromosome and mitotic machinery targets (*e.g.* centromeres and kinetochores, respectively) [3,11].

2.3. Which classes of chemical genotoxins can or cannot be detected by the lymphocyte CBMN assay as used for human bio-monitoring?

In principle, the CBMN assay can only detect genotoxin exposure when used in human bio-monitoring if (i) micronuclei are induced *in vivo* and are not eliminated by apoptosis or (ii) if the DNA damage in the G0 lymphocytes is not repaired and can be converted to MN *ex vivo* during their first mitotic division or (iii) if the *in vivo* exposure causes long lasting damages to the relevant chromosome and mitotic machinery targets. There is *in vitro* evidence that some chemicals which predominantly induce DNA adducts (*e.g.* methylnitrosourea) may not be able to induce MN if lymphocytes are exposed in G0 only, however, it is not yet clear whether this applies to all chemicals that predominantly induce DNA adducts or all types of adducts (*e.g.* small *versus* bulky adducts). Furthermore, it is improbable that a chemical will only induce DNA adducts and not affect other cellular structures and DNA maintenance pathways which, if impaired, may also induce MN formation. There is clearly a need to determine which of the chemicals that predominantly induce DNA adducts will not induce MN in the conventional CBMN assay protocol for bio-monitoring if exposure occurs only in G0.

2.4. Can the conventional lymphocyte CBMN assay also be used to measure in vivo *induced MN that are already in the cells?*

MN were first described in human erythrocytes more than 100 years ago and later shown to be due to chromosome aberrations in normoblasts in the bone marrow caused by folate and/or vitamin B12 deficiency [1,39–42]. It is known that MN can also be induced in lymphoblasts in the bone marrow and lymph nodes during lymphocyte proliferation [1,41–43]. Therefore a genotoxic exposome in the body is expected to induce MN *in vivo* in lymphocytes. In a paper on the effects of chronic exposure to radiation we showed that MN in non-divided lymphocytes in the CBMN assay were also a good indicator of exposure when compared to MN in cytokinesis-blocked binucleated cells [44]. We, in fact, proposed that MN should also be scored in non-divided mononucleated cells within the CBMN assay and that the most efficient time to do this could be 24 h after PHA stimulation when the cytoplasm is enlarged making it easier to observe the MN in cells [6,44,45]. Whether scoring MN in non-divided cells is best done at 0, 24 h or at 72 h when the CBMN assay culture is typically harvested has not yet been tested. Perhaps mononucleated cells with MN may undergo apoptosis as they progress through the cell cycle *ex vivo* or may reappear if they complete nuclear division [45]. Live cell imaging studies suggest that cells with MN tend to either not proliferate or terminate apoptotically but those that do divide tend to generate daughter cells with MN [46]; whether this also applies to human lymphocytes *in vivo* or *ex vivo* has not yet been determined.

2.5. Can the CBMN assay protocol be modified to improve sensitivity to chemically induced DNA lesions that are not repaired or efficiently converted to micronuclei?

There are essentially three ways that the CBMN assay protocol has been modified or is being modified to capture DNA lesions that are not efficiently converted to micronuclei:

(i) As indicated above it is possible to convert excision repairable DNA lesions to DNA strand breaks within one cell cycle by treating with ARA-C (an inhibitor of the gap filling step of base excision repair) during G1 phase post PHA stimulation *ex vivo* in culture We have shown that this approach increases the sensitivity to DNA damage from ionising radiation 1.8-fold, UV 40-fold and in the case of MNU from undetectable to a steep dose-response curve. The mechanism was verified because, as predicted, the majority of ARA-C induced MN were due to acentric chromosome fragments [23,24]. However, it is not yet clear whether unrepaired DNA adducts induced *in vivo* can also be efficiently converted to DNA strand breaks and MN *ex vivo* using the ARA-C protocol.

(ii) DNA probes and/or antibodies can be used in the CBMN assay to measure mal-segregation of chromosomes between nuclei of binucleated cells, telomere and centromere content of nuclei and micronuclei, as well as a variety of DNA damage response proteins and DNA adducts all of which can provide additional information on chemically induced genomic insults [3,6,11].

(iii) Because many genotoxic chemicals also induce necrosis, apoptosis and cytostasis to varying degrees it is also important to score these events to obtain a complete picture of the toxicity profile and also capture any cells with DNA damage that have become apoptotic [3,6]. These cell types and the ratio of mononucleated, binucleated and multinucleated cells to measure the nuclear division index are included in the cytome version of the CBMN assay. The extent to which these biomarkers correlate with DNA adduct load and other DNA damage events is not clear because it has not been methodically investigated.

2.6. Does the inclusion of other nuclear anomalies such as nucleoplasmic bridges and nuclear buds in the "cytome" version of the method improve the sensitivity of the CBMN assay for detecting in vivo *chemical genotoxin exposure?*

The CBMN cytome assay (Fig. 3) also includes additional biomarkers of chromosomal instability such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). NPBs may arise from dicentric chromosomes or chromatids caused by mis-repair of DNA breaks or due to telomere end fusions caused by telomere dysfunction; in addition NPBs may also be caused by incomplete separation of chromatids during the metaphase to anaphase transition [3,47]. NBUDs originate from nuclear removal of unresolved DNA repair complexes and excess amplified DNA; they may also be induced by breakage of NPBs [3]. NPBs and NBUDs have widened the spectrum of DNA damage events detectable by the CBMN assay but the classes of chemicals that efficiently induce these biomarkers are yet to be defined. The use of NPBs in the CBMN cytome assay is unique amongst biomarkers of genotoxicity because it is the only method that can measure defects in chromatid separation during anaphase which leads to chromosomal instability [3,6]. The growing number of studies using the comprehensive CBMN cytome assay will make it possible to identify the relative sensitivities of MN, NPB and NBUD to different classes of chemical genotoxins and their profile uniqueness with respect to chemical type. For example it was shown that the NPB/MN ratio was much higher in WIL2-NS cells after activated neutrophil exposure than it was for superoxide or hydrogen peroxide [48]. A study of workers exposed to vanadium pentoxide showed that NPBs were increased to a much greater extent than

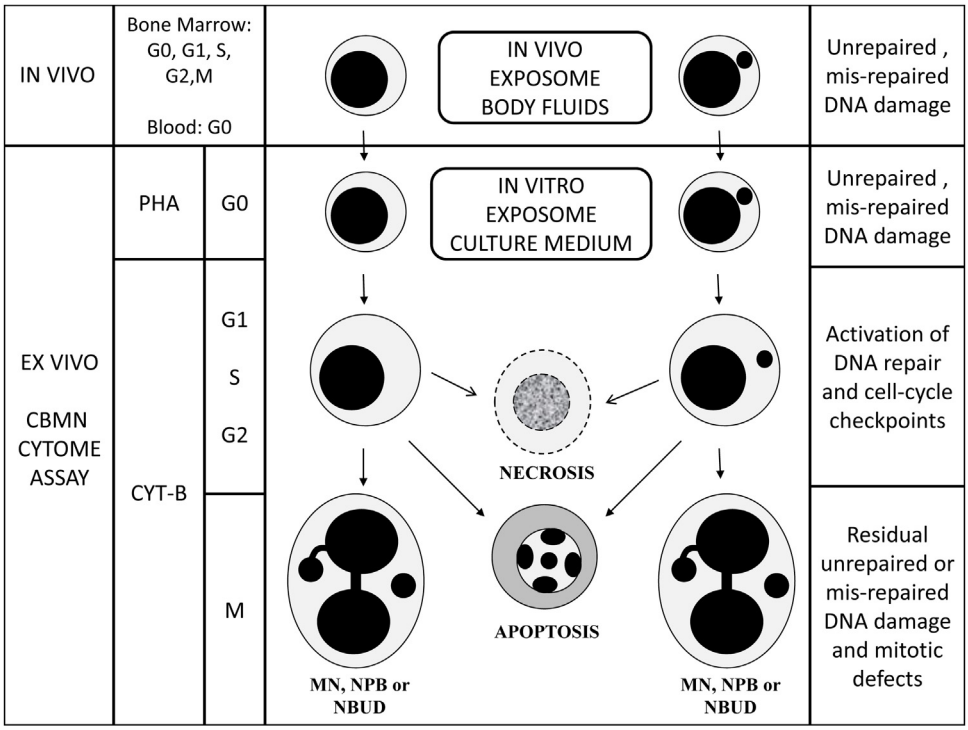

Fig. 3. The stages in the lymphocyte CBMN cytome assay when MN and other nuclear anomalies can be measured in cells exposed *in vivo* to genotoxicants. Measurements in mononucleated cells prior to completion of mitosis and after mitosis in binucleated cells allows discrimination between MN expressed *in vivo* and those expressed *ex vivo* respectively. CYT-B, cytochalasin-B; MN, micronuclei; NBUD, nuclear buds; NPB, nucleoplasmic bridges; PHA, phytohaemagglutinin.

MN in the exposed group relative to controls [49] and NPBs in lymphocytes of smokers were associated with lung cancer to a much greater extent as compared to MN [50,51].

2.7. Which other complementary genome/chromatin damage biomarkers should be used in combination with the CBMN cytome assay to enhance detection of chemical genotoxin exposure?

The CBMN cytome assay, being a multiple biomarker system, can capture many types of damages induced by the myriad of genotoxic chemicals and their combinations. Nevertheless, it is advisable to consider using a high content analysis (HCA) approach in the application of the CBMN cytome assay by combining it with molecular detection systems to also measure presence or absence of centromere/s or telomere/s within MN, NPB or NBUD, as well as DNA damage response proteins (*e.g.* γH2AX as a biomarker of DNA strand breaks), DNA adducts (*e.g.* 8-OHdG as a biomarker of oxidative DNA damage), DNA methylation and telomere content within the nuclei. In addition it has recently been shown that gene expression profiling could also be used to identify the metabolic and DNA maintenance pathways associated with the CBMN assay biomarkers which can then provide a clue of which aspect of the exposome was the likely causative factor [52,53]. Therefore, there is a need to reconsider whether a HCA CBMN cytome assay that may or may not include an ARA-C step will provide a better assessment of chemical genotoxin exposure and its genetic consequences.

3. Misconceptions and knowledge gaps about the sensitivity of the CBMN assay for detecting exposure to chemical genotoxins

The questions and considerations described above should not be confused with misconceptions that have emerged recently about the use and reliability of the lymphocyte CBMN assay for measuring DNA damage caused by *in vivo* exposure to chemical genotoxins [54]. Uncertainties about the reliability of results obtained with the lymphocyte CBMN assay to measure *in vivo* DNA damage induced by chemicals may be due to (i) a paucity of systematic critical reviews of the vast literature and data on the use of CBMN assay in population bio-monitoring of genotoxic chemical exposure and/or (ii) limited knowledge of the varied and multiple mechanisms by which a genotoxic chemical may directly or indirectly cause damage to DNA and the mitotic apparatus that can lead to MN formation and other associated nuclear anomalies in lymphocytes *in vivo*. It has therefore become necessary to discuss the apparent knowledge gaps associated with these misconceptions that have emerged recently in the literature:

3.1. Misconception #1: the CBMN assay excludes MN being scored in non-divided cells

The CBMN assay as originally conceived has, since 15 years ago, evolved into a CBMN cytome assay that apart from scoring MN in binucleated cells also includes scoring MN in non-divided mononucleated cells as well as apoptotic cells to take account of their possible elimination by programmed cell death. This was first recommended by Fenech et al. [44] and subsequently by Kirsch-Volders and Fenech [45]. By using this protocol MN that are already expressed *in vivo* are also captured in the assay. There are now several published studies that use this approach [44,55–58]. For example, Fucic et al. [55] showed that the MN frequency in binucleated lymphocytes *ex vivo* in newborns was significantly lower than in their mothers but higher in mononuclear lymphocytes suggesting a higher susceptibility for MN induction *in vivo* but a lower propensity for MN expression *ex vivo* in newborns relative to their mothers. The reasons for such differences are not known but the results suggest the possibility of increased sensitivity of the newborn to MN induction *in vivo* induced by metabolic/oxidative stress during the late stages of pregnancy particularly if the mother has an elevated body mass index [55,56]. This example and the others mentioned above highlight the opportunity of a more comprehensive assessment of the origins of DNA damage by scoring MN that are already present in lymphocytes and those expressed *ex vivo* in once-divided binucleated cells.

3.2. Misconception #2: MN produced in vivo do not substantially contribute to MN frequency measured in binucleated lymphocytes in the ex vivo CBMN assay

The only reliable way to know whether MN produced *in vivo* contribute to MN formation in binucleated cells in the *ex vivo* CBMN assay is to follow, by live-cell imaging, the fate of lymphocytes that already contain MN prior to mitogen stimulation. No such studies have yet been performed in lymphocytes. Even if these cells did not survive and became apoptotic they would still be detected as apoptotic cells in the CBMN cytome assay [6]. Furthermore, if they are delayed in undergoing mitosis they would be detected as MN in mononucleated cells anyway [44,45].

3.3. Misconception #3: the sensitivity of the CBMN assay for detection of MN in binucleated cells is diminished because cytochalasin-B is added late during the culture period so that the binucleated cells scored do not always represent cells that have completed one cell cycle only

In the CBMN lymphocyte assay cytochalasin-B (CYT-B) is added 44 h after mitogen stimulation to block cytokinesis, so that cells that have completed one nuclear division can be easily identified by their binucleate appearance and then scored for MN and other nuclear anomalies. There is as yet no evidence to suggest that adding CYT-B earlier than 44 h improves the sensitivity of the CBMN assay. A protocol based on adding CYT-B at 24 h post PHA stimulation and harvesting at 48 h showed that only 5% of cells were accumulated as binucleated cells during this period [59] which is a very small fraction compared to binucleated cell frequencies of between 30% and 50% for protocols based on adding CYT-B at 44 h and harvesting at 72 h. Furthermore, cells with DNA damage may experience cell cycle checkpoint delay, therefore they are more likely to undergo mitosis at a later rather than an earlier time-point. At best only 5% of binucleated cells would have undergone more than one division and there is no evidence to suggest that this would substantially alter the MN frequency in binucleated cells.

3.4. Misconception #4: the delay in adding CYT-B means that damaged cells can be eliminated by apoptosis and/or DNA damage induced in vivo can be repaired prior to the production of a MN in the presence of CYT-B. This may render the CBMN assay to be insensitive

As indicated above the CBMN cytome assay includes scoring of apoptotic cells, therefore DNA damaged cells are not excluded from the CBMN cytome assay score. The ratio of apoptotic cells to cells that divide and express DNA damage can be a useful indicator of an individual's capacity to trigger apoptosis and therefore may indicate cancer susceptibility. DNA repair is upregulated when lymphocytes enter G1 phase [23,24]. By allowing repair to occur prior to accumulation of binucleated cells the CBMN assay provides a better indication of the actual genotoxic risk to the cells *in vivo* because it takes into account the capacity of the cell to repair the DNA lesions induced by the exposome which they

experience *in vivo*. Furthermore, it also takes into account mis-repair or defects in repair which could induce chromosome aberrations that lead to MN formation [14–16]. This integrated "genotoxic risk" measurement would differ from that of a CBMN assay where inhibition of DNA repair during the *ex vivo* step would be experimentally induced to increase conversion of excision-repairable DNA adduct lesions to MN and thus reflect more closely "exposure level" [23,24]. Both can be interesting but the choice of the protocol design should also consider this important hazard *versus* risk perspective.

3.5. Misconception #5: a comparison with the in vitro CBMN assay used for genotoxicity testing leads to the conclusion that it is unlikely that DNA damage induced in vivo is the cause of increased MN frequencies in binucleated cells after occupational or environmental exposure to genotoxic chemicals

The *in vitro* CBMN lymphocyte assay performed according to the OECD guideline [2] can test the effects of genotoxic chemical exposure at all stages of the cell cycle as occurs in *in vivo* exposure in those tissues of the body where lymphocytes are generated as a result of cell division and maturation of precursor cells (*e.g.* bone marrow and spleen). However, results from *in vitro* assays may not accurately predict *in vivo* effects because *in vitro* treatment with chemicals does not replicate all of the complexities of *in vivo* exposure such as absorption, metabolic activation or detoxification and excretion of the chemical by other tissues in the body. The lymphocytes in blood may already contain MN induced *in vivo* in the dividing lymphocyte populations and these are captured in non-divided mononucleated cells that are scored in the CBMN cytome assay. Furthermore, because a large proportion of lymphocytes are long-lived they also accumulate DNA damages (*e.g.* DNA adducts, DNA strand breaks) depending on the *in vivo* exposome and these damages can also lead to the induction of MN, *ex vivo* after one cell division in culture, which are scored in the cytokinesis-blocked binucleated cells in the CBMN assay. The exposome in an *in vitro* system consists simply of the composition of the culture medium, the test chemical and any solvent used; however effects could vary depending on the nutritional composition of the medium which supplies cofactors for DNA replication and repair and may vary from *in vivo* effects because the nutritional composition of typically used culture media varies substantially from the nutritional profile of body fluids such as plasma [60]. Similarly the genotoxic effects of a chemical *in vivo* may vary depending on the nutritional and metabolic status of the subjects investigated and could be further complicated by other concurrent genotoxin exposures that may interact with the chemical exposure being investigated [3,56,60]. Therefore, *in vivo* exposure to a chemical genotoxin will, ultimately, contribute to the totality of the genotoxic *in vivo* exposome which induces the various other DNA lesions already present that can also lead to MN formation in lymphocytes *in vivo* and/or *ex vivo*.

A PubMed search for biomonitoring studies investigating lymphocyte MN frequency in populations exposed to chemical genotoxins yields hundreds of publications, the majority of which indicating significant increases in MN frequency in cohorts exposed to a wide range of genotoxic chemicals (*e.g.* pesticides, benzene, formaldehyde, vinyl chloride, polycyclic aromatic hydrocarbons, heavy metals cytotoxic drugs). For example, since 1993, seventeen *in vivo* studies of formaldehyde exposure in humans, in different settings, were published [27,61–76]. All of these studies, except two, reported significant increases in the frequency of MN in the lymphocyte CBMN assay for the exposed groups relative to the controls. Furthermore four of five studies showing an increase in oral and/or nasal MN frequency in those exposed to formaldehyde also showed a significant increase in MN frequency in the lymphocyte CBMN assay in these groups relative to controls [61,62,67,68,73]. These data clearly support the notion that the CBMN assay is sensitive to *in vivo* exposure from genotoxic chemicals such as formaldehyde. Furthermore, it is well documented that mutagens which induce MN in lymphocytes in the CBMN assay are also likely to initiate cancer [2,5,7,50–53]. In addition, proof for the assumption that MN reflect DNA damage in humans comes from positive findings in several MN studies with lymphocytes which were performed with population groups with occupational and lifestyle related exposures to known genotoxins and/or with certain DNA maintenance pathologies (*e.g.* DNA repair and DNA methylation deficiencies) that are known to be associated with increased cancer rates [5,8,14,25–42,48–52]. In this context it is also notable that the epidemiological evaluation of data from 6718 subjects from 10 countries indicates that MN rates in lymphocytes are a predictive biomarker for human cancer risks [77].

However, a systematic assessment of all published peer-reviewed studies, for each class of chemicals, is required to determine effect sizes, statistical significance and consistency across investigations depending on the quality of study designs. At this stage, the available published data has not been properly analysed to determine (i) whether the CBMN cytome assay, as currently performed, can detect all types of chemical genotoxin exposures and their associated genetic hazard or (ii) the exposure levels that may be below the detection limit of the assay. Increases in MN frequency in the lymphocyte CBMN assay reported in a multitude of chemical occupational exposure studies in the peer-reviewed literature are more likely to be accepted by experts in the field when the mechanisms by which chemicals might induce MN in lymphocytes *in vivo* or *ex vivo* are properly elucidated and proven. It is also reasonable to consider whether studies reporting positive results were properly controlled for other factors such as diet, life-style, gender and age which may also contribute to the MN frequency index [7]. Finally the high positive correlation ($R^2 = 0.74$) of MN induction by chemical genotoxins in the occupational setting observed between lymphocytes in the CBMN assay and buccal cells [78] adds support to the evidence that MN scored in lymphocytes in the *ex vivo* CBMN assay do in fact represent DNA damage induced *in vivo* because MN in buccal cells are expressed entirely and solely *in vivo*.

4. Is it possible to further improve the CBMN cytome assay for detecting exposure to genotoxic chemicals and to measure the genetic hazard/risk associated with such exposures?

Because MN and associated nuclear anomalies can be induced by different exposures such as nutritional deficiency, ionising radiation and direct- or indirect-acting chemical genotoxins it is not possible to be absolutely certain how they may have been induced. However it is possible to use the CBMN cytome biomarker profile to determine the mechanism by which they were induced. For example exposure to ionising radiation typically increases the frequency of MN and NPB and the majority of induced MN are centromere negative [3,4,14]. In contrast chemicals which induce chromosome malsegregation result in the formation of MN that are centromere positive and the daughter nuclei have abnormal numbers of chromosomes (even in the absence of MN induction) which can be detected using chromosome specific centromere probes. Another approach is to use molecular probes to detect specific DNA adducts (*e.g.* antibodies to 8-oxoguanine, or polycyclic aromatic hydrocarbon adducts) within the nuclei or DNA damage response proteins that are specific to the mechanism of action of the genotoxin. For example, phosphorylated γH2AX can be used to measure the formation of double stranded DNA breaks within the nuclei of cells scored although this biomarker is also

affected by multiple exposures including metabolic disorders such as obesity [79]. In a recent study with heavy ions it was possible to show that micronuclei resulting from such an exposure specifically express γH2AX and SMAD7 [80]. It is possible that a selection of DNA damage response proteins expressed in MN and the nucleus of the cell they inhabit could shed more light on exposure to specific genotoxins. Another important aspect to consider is also the possibility of detection of chromosome shattering (chromothripsis) occurring within MN due to incomplete DNA replication and ligation of Okazaki fragments during the mitotic cycle. The induction of this defect is considered a key mechanism in the formation of highly rearranged chromosomes (chromoanagenesis) typically observed in cancers [81,82].

Given the relative non-specificity of MN induction with respect to genotoxin exposure it is reasonable to expect that the CBMN cytome assay on its own may not provide sufficient information on the full extent of genotoxin exposure. On the other hand, the sensitivity of the assay to diverse genotoxins acting through various mechanisms, together with its capacity to also measure NPBs, NBUDs, necrosis, apoptosis and cytostasis in cytome mode, makes it one of the more suitable techniques to capture the genetic and cytotoxic hazard posed by multiple genotoxins and their interactions within the exposome of a human body as well as the impact of susceptibility caused by genetic defects and nutritional deficiencies/excesses.

Another aspect to consider is that in the lymphocyte CBMN cytome assay, apart from measuring MN that are already present within lymphocytes, it also measures DNA damages accumulated in the body that are readily converted into MN when dividing *ex vivo* in culture [3–6]. Because the *ex vivo* culture should reflect what might happen in the body if the damaged lymphocytes were to divide *in vivo* it may be worthwhile exploring whether culturing of lymphocytes in medium that uses plasma from the blood of the individual being tested instead of foetal bovine serum, and has physiological amounts of micronutrients required for DNA repair may further enhance sensitivity of the assay. In fact the micronutrient composition of culture medium is non-physiological if compared to the composition of human serum or plasma and efforts are underway to develop culture media that better represent the human body fluid conditions [60].

As has been the case with *in vitro* genotoxicity testing we are at a stage when it has become important to reconsider what is the best combination of genotoxicity assays to measure (i) the extent of exposure to genotoxins within the human body and (ii) the genetic hazard and disease risk posed by exposomes of varying complexity.

5. A road map to resolve the knowledge and technological gaps in the use of the lymphocyte CBMN cytome assay for detection of chemical genotoxin exposure and the induced genetic hazard/risk

The HUMN project, originally established in 1997 (www.humn.org), has over the past 16 years contributed greatly to improving the application and understanding of lymphocyte and buccal cell micronucleus assays in human biomonitoring and determining the health risk associated with elevated MN frequencies. Based on this experience we propose the following key steps to resolve the knowledge and technological gaps in the use of the lymphocyte CBMN cytome assay for the detection of chemical genotoxin exposure and the induced genetic hazard/risk:

(1) There are hundreds of papers describing studies with the CBMN and/or CBMN cytome assay of medical, environmental and occupational exposure to single chemical genotoxins and complex mixtures. With the exception of exposure to pesticides [8] these studies have not been previously systematically reviewed. We propose that a systematic review of all of these studies by experts in the field be performed to determine the quality of study designs, the adequacy of statistical analyses, and the extent to which the lymphocyte CBMN cytome assay biomarkers were associated with exposure to genotoxic agents depending on class of chemical and dose. The extent to which genotype affects the effect size and the plausibility of an effect depending on mechanism should also be assessed when possible. Furthermore, in studies, where other genotoxicity assays were used, it will be useful to also provide an estimate of the effect size of the CBMN cytome assay biomarkers relative to DNA damage measured by other assays. A series of systematic reviews, one for each class of chemical genotoxins, of studies which have investigated the association of *in vivo* exposure in humans with MN, NPB and NBUD induction measured using the lymphocyte CBMN cytome assay is recommended.

(2) It is becoming increasingly evident that a genotoxic chemical may have multiple targets other than DNA that can directly or indirectly also cause chromosomal instability and mitotic malsegregation events that can lead to formation of MN, NPBs and NBUDs (Fig. 1). Therefore, it is now important to review the literature to obtain new insights on the mechanisms by which different classes of chemicals may induce MN, NPB and NBUDs *in vivo*.

(3) Finally, it should be useful to conduct a series of workshops to explore how the lymphocyte CBMN cytome assay may be further improved to increase sensitivity to chemical genotoxins and discuss the best combination of genotoxicity assays to comprehensively assess the genome (DNA) damage risk from single and/or multiple exposures to chemical genotoxins. For example a recent review on traffic-associated genotoxicity biomarkers highlighted the importance of an integrated approach using validated complementary diagnostics of DNA damage at the chromosomal and molecular level to provide the strongest mechanistic evidence that exposure to traffic fumes is likely to be carcinogenic [83]. Achieving a consensus on the best approach(es) to measure exposure to chemical genotoxins and associated genetic hazard using the lymphocyte CBMN cytome assay in conjunction with other techniques should be a high priority.

One may also consider whether some of the questions about the suitability of the lymphocyte CBMN cytome assay for detecting *in vivo* exposure to a wide range of chemical genotoxins in humans may be indirectly explored by controlled acute or chronic exposure studies in rodents which are not ethically possible in humans. In fact a lymphocyte CBMN *ex vivo* assay to detect *in vivo* exposure to chemical genotoxins and ionising radiation was developed and validated for the mouse more than 20 years ago [84,85]. It would be interesting to test whether this approach, or use of MN assays in target organs such as the lung [86], is more sensitive than the conventional erythrocyte MN assay which, unlike lymphocytes which circulate throughout the body, is entirely dependent on the chemical genotoxin reaching the bone marrow. Nevertheless, because of the dearth of available evidence, one cannot assume that data from controlled lymphocyte CBMN assays in rodents will reliably predict results in humans following *in vivo* exposure to chemical genotoxins.

6. Conclusion

In conclusion, the lymphocyte CBMN cytome assay has a great potential to serve as a reliable biomarker for genetic damage in biomonitoring studies of chemical genotoxin exposure, essentially

because it allows a risk analysis at the individual cell level and combines assessment of both structural and numerical chromosome aberrations. The growing interest in this method and the wealth of data that has been accumulated so far has stimulated the need to re-evaluate the use of MN and other biomarkers within the lymphocyte CBMN cytome assay as quantitative indicators of exposure to chemical genotoxins and the genetic hazard/risk they may cause. This review has identified some important misconceptions as well as knowledge and technological gaps that need to be addressed to make further progress in the proper application of this promising technique and enable its full potential to be realised.

Conflict of interest

There is no conflict on interest.

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