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Inclusion of micronuclei in non-divided mononuclear lymphocytes and necrosis/apoptosis may provide a more comprehensive cytokinesis block micronucleus assay for biomonitoring purposes

Micheline Kirsch-Volders[2] and Michael Fenech[1]

Vrije Universiteit Brussel, Laboratorium voor Cellulaire Genetica, Pleinlaan 2, 1050 Brussels, Belgium and [1]CSIRO Health Sciences and Nutrition, PO Box 10041, Gouger Street, Adelaide, SA 5000, Australia

Human biomonitoring of early genetic effects requires accurate, sensitive and, if possible, easy and not too time-consuming methodologies to assess mutations. One of the most promising methodologies at the present time is the cytokinesis block micronucleus (MN) assay (CBMN), which detects both chromosome breakage and chromosome loss in once-divided binucleated (BN) cells. Many studies have been published with this methodology, but before its extensive application is recommended, it is necessary to evaluate its strengths and limitations. Recently, Fenech *et al.* reviewed the advantages of the CBMN assay for biomonitoring purposes. However, up to now information present in mononucleated (MONO) cells has rarely been taken into account, although it might be complementary to that assessed in BN cells. Indeed, MONO cells should indicate damage which was present *in vivo* before the start of culture and BN cells may contain pre-existing micronuclei (MNi) plus lesions which are expressed as MNi during *in vitro* culture. To address this question, the objectives of this paper were as follows. (i) To situate the CBMN assay in a historical and mechanistic perspective. (ii) To consider whether impaired mitotic capacity *in vitro* may be responsible for false negative biomonitoring studies if MN in MONO cells are not taken into account in the CBMN test. The following factors were considered: division delay for repair and mitotic block, *in vitro* apoptosis and necrosis of damaged cells, mitotic slippage and correlation between MN expression *in vitro* versus *in vivo*. (iii) To analyse the factors which may cause a negative result in the CBMN assay in biomonitoring when exposure to specific genotoxins is evident. The specific effects of aneugens and of adaptive responses to chronic low level exposure were examined. (iv) To compare the sensitivity of MONO and BN cells in relation to the genotoxic mechanism. (v) To propose an adequate sampling scheme to study MN in both MONO and BN cells. It was concluded that a more comprehensive assessment of DNA damage may be achieved if the CBMN assay includes measures of: (i) MNi in MONO cells; (ii) MNi in BN cells; (iii) apoptotic cells; (iv) necrotic cells. It is probable that the 24 h post-phytohaemagglutinin time point may be the optimal time to assess the frequency of MNi in MONO cells, apoptotic cells and necrotic cells. It is also practical to include these measures when scoring MNi in BN cells after cytokinesis block.

Introduction

It is becoming increasingly evident that an increased rate of DNA damage and chromosome breakage or loss is an important risk factor for elevated risk for cancer (Hagmar *et al.*, 1994, 1998; Bonassi *et al.*, 2000) and possibly other ageing degenerative diseases such as Alzheimer's disease (Migliore *et al.*, 1999). Human biomonitoring of early genetic effects requires accurate, sensitive and, if possible, easy and not too time-consuming methodologies to assess mutations. One of the most promising methodologies at the present time for assessing DNA damage is the cytokinesis block micronucleus (CBMN) assay, which detects both chromosome and genome mutations in binucleated (BN) cells. Many studies have been published with this methodology (reviewed in Fenech *et al.*, 1999a), but before its extensive application is recommended, it is necessary to evaluate its strengths and limitations.

The micronucleus (MN) assay in human biomonitoring studies is mainly applied to peripheral blood lymphocytes (for reviews see Fenech, 1998; Fenech *et al.*, 1999a; Kirsch-Volders *et al.*, 1997, 2000; Surraales and Natarajan, 1997) and to a lesser extent in epithelial cells. This assay is being used to: (i) compare genetic damage rate between populations exposed to different environmental, occupational and lifestyle factors (Fenech *et al.*, 1999a); (ii) assess differences in radiosensitivity between individuals at risk for cancer both as a predictor of cancer risk as well as for optimization of radiotherapy (Scott *et al.*, 1998); (iii) assess the genotoxic potential of new chemicals produced by the agrochemical and pharmaceutical industries (Kirsch-Volders *et al.*, 2000).

Micronuclei (MNi) may originate from an acentric chromosome fragment or whole chromosomes lost from the metaphase plate and provide therefore a measure of both chromosome breakage and chromosome loss. An additional advantage of this end-point is that it can be scored relatively easily and in a range of cell types relevant for human biomonitoring, such as lymphocytes, fibroblasts and exfoliated epithelial cells, e.g. oral, nasal or urothelial mucosa (for a review see Fenech *et al.*, 1999a). By its very nature, a MN requires cell division to take place after damage induction and therefore knowledge of the division kinetics of the studied tissue is a prerequisite to correctly interpret the observed MN frequencies: MNi observed in exfoliated cells (epidermis of skin, urinary bladder, mouth or nasal mucosa) are not induced when the cells are in the upper layer, but when they are in the basal layer. The kinetics of MN expression in peripheral blood erythrocytes, both in mice and humans, has been well characterized, but application is limited in humans to splenectomized subjects as the spleen removes micronucleated (MNed) erythrocytes (MacGregor *et al.*, 1980; Schlegel *et al.*, 1986).

The CBMN methodology (Fenech and Morley, 1985), based on inhibition of the actin furrow during anaphase by cytochalasin B, allows discrimination between cells which did not divide (mononucleated cells) after treatment from those which divided once (binucleated cells) or more (multinucleated cells) *ex vivo*/*in vitro* and stimulated new applications of the MN test for both *in vitro* genotoxicity testing and human biomonitoring

[2]To whom correspondence should be adressed. Email: mkirschv@vub.ac.be

studies. In particular, the *in vitro* MN test on lymphocytes and cell lines received a lot of attention and is now being considered as an advantageous alternative for the *in vitro* chromosome aberration test (for reviews see ICH, 1995, 1997; UKEMS, 1989, 2000; Kirsch-Volders, 1997; Kirsch-Volders *et al.*, 2000). In combination with molecular probing techniques for specific chromosome regions and/or cytotoxicity tests, the method permits concurrent scoring of additional end-points: chromosome non-disjunction, chromosome rearrangement (nucleoplasmic bridges), excision repaired sites (ARA-C protocol), apoptosis and necrosis (for reviews see Fenech, 1997; Kirsch-Volders *et al.*, 1997).

Micronuclei in non-divided mononucleated cells

Recently, Fenech *et al.* (1999a) reviewed the advantages of the CBMN assay for biomonitoring purposes. However, up to now information present in mononucleated (MONO) cells was rarely taken into account, although it might be complementary to that assessed in once-divided BN cells that are typically scored in the CBMN assay. Indeed, a MN in a MONO cell should indicate chromosome damage which was present *in vivo* before the start of culture and BN cells may contain pre-existing MNi plus lesions which are expressed as MNi during *in vitro* culture. To address this question, the objectives of this paper are first to set the CBMN assay in a historical and mechanistic perspective, then analyse the advantages of scoring MONO cells in addition to BN cells and finally propose an adequate sampling scheme to study MNi in both MONO and BN cells.

The possibility of *in vitro* subcultivating human lymphocytes from populations to be analysed after *in vivo* exposure led to the use of an *ex vivo*/*in vitro* CBMN test where the frequencies of BN cells are scored to assess the replicative capacity of cells and the frequencies of BN cells with micronuclei (MNBN) to estimate the DNA lesions which were present and expressed as MNi through the first *in vitro* mitosis. Using inhibitors of the excision gap filling step it was also shown that excision repairable adducts on DNA could be converted to MNi and quantified in this assay (Fenech and Neville, 1992). This approach did not take into account MNi that may have already been present in lymphocytes prior to culture or MNi expressed in cells that were not cytokinesis blocked. In fact, MNi in MONO cells indicate chromosome breakage damage before the blood was sampled and MNi in BN cells may originate from pre-existing MNi plus lesions that are expressed as chromosome breaks during replication. It is important, however, to note possibilities which are fundamental for the assessment of MNi in MONO cells: (i) one cannot control *in vivo* cell division kinetics so that MNi in MONO cells may be less reliable than MNi in BN cells; (ii) it becomes necessary to consider that the MONO cells scored in the CBMN test might in some cases represent either cells which did not divide, cells which replicated DNA but escaped nuclear division or cells which divided but escaped the cytochalasin B block and, moreover, that some MONO cells never reached the stage of nuclear division because they were at a very early stage of lesion-induced apoptosis or necrosis; (iii) MONO cells with MNi may have a low probability of becoming BN cells with MNi because of possible inhibition of nuclear division. Therefore, a MNBN is more likely to be an indication of strand breaks or other DNA damage accumulated while lymphocytes are in the G_0 phase *in vivo*. For these reasons, MNi in MONO cells and MNi in BN cells are likely to be different but complementary.

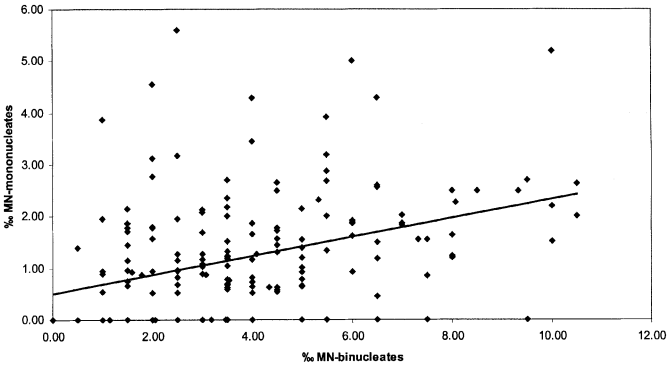

Fig. 1. Correlation between MN frequency in MNed cells and MN frequency in BN cells at 72 h in the CBMN assay. $r = 0.361$, $P < 0.0001$. Results from Elhajouji *et al.* (1998).

For the purposes of biomonitoring occupational or environmental exposure to mutagens the comparison of MNi frequencies in MONO cells might provide interesting additional information. Indeed the MNi frequencies in MONO cells may give an estimation of the genome instability accumulated over many years in stem cells and circulating lymphocytes, while the MNi in BN cells additionally provide a measure of the lesions which have accumulated in the DNA since the cells last replicated *in vivo*.

Fenech *et al.* (1997) compared the frequencies of MNi in erythrocytes, non-divided MONO lymphocytes and cultured cytokinesis-blocked BN cells in children from the Chernobyl disaster. They concluded that a differential level, and possibly a different spectrum, of damage was observed in these different types of cells. Elhajouji *et al.* (1998) analysed the background frequencies of MNi in MONO versus BN cells in a control population of 240 healthy donors (230 men and 10 women non-exposed to known mutagens) (Figure 1). The frequencies of MNi in MONO ranged from 0 to 5.60‰ with a median value of 0.99‰ and for MNi in BN cells from 0 to 10.5‰ with a median value of 3.5‰. The correlation coefficient between the frequencies of MNi in MONO cells and the frequencies of MNi in BN cells was $r = 0.366$ ($P < 0.0001$). The frequency distribution showed that 100% of analysed control subjects had between 0 and 5‰ MNi in MONO cells and 50% had between 0 and 1‰ MNi in MONO cells. For MNi in BN cells, the frequency distribution showed that 80% of the analysed controls had between 0 and 5‰ MNi in BN cells but only 9% had between 0 and 1‰ MNi in BN cells. The frequency of MNi in BN cells was thus in general higher than the frequency of MNi in MONO cells in control subjects. The frequency of MNi in both BN and MONO cells was age dependent ($r_{MONO} = 0.123$, $P_{MONO} < 0.05$; $r_{BN} = 0.223$, $P_{BN} < 0.002$). Because the MONO cells were scored at 72 h post-mitogen stimulation one cannot be certain that the MONO cells scored were non-divided cells or cells that escaped the cytochalasin B block (although the latter is unlikely given that the concentration used was optimal for cytokinesis blocking). A potentially better alternative approach for scoring MNi in non-divided T lymphocytes is suggested below.

The other objectives of this paper are as follows.

- To consider whether impaired mitotic capacity *in vitro* may be responsible for false negative biomonitoring studies if MNi in MONO cells are not taken into account in the

CBMN test. The following factors are considered: division delay for repair and mitotic block, *in vitro* apoptosis and necrosis of damaged cells, mitotic slippage and correlation between MNi expression *in vitro* versus *in vivo*.
- To analyse the factors which may cause a negative result in the CBMN assay in biomonitoring when exposure to specific genotoxins is evident. The specific effects of aneugens and of adaptive responses to chronic low level exposure are examined.
- To compare the potential sensitivity of MONO and BN cells in relation to the genotoxic mechanism.
- To propose an adequate sampling scheme to study MNi in both MONO and BN cells.

Factors influencing the mitotic capacity

Physical and chemical mutagens, hypoxia and other cellular stress factors are known to trigger p53, which in turn orientates the cell to different outcomes depending on the phase of the cell cycle, the type of lesion and the importance of the damage. The major alternatives in the presence of genotoxic lesions are toleration of DNA damage, generation of an abnormal base sequence for the sake of cell survival, cell cycle arrest to allow DNA repair or apoptosis/necrosis. Binding of toxic substances to non-DNA targets may induce apoptosis through several pathways, necrosis or mitotic slippage. These many responses of cells to environmental factors can significantly modify the frequencies of cells which undergo mitosis at a given time in culture and therefore the frequencies of BN cells at harvest. In the following the factors capable of influencing mitotic capacity will be addressed.

Division delay for repair and mitotic block

DNA repair can be defined in a general sense as a range of cellular responses associated with restoration of the genetic instructions as provided by the normal primary DNA sequence (for a review see Lindahl and Wood, 1999). More than 70 human genes are directly involved in the five major pathways of DNA repair. Cell cycle regulation is closely coupled with DNA damage responses (for a review see Yu *et al.*, 1999). Most of the repair activities are S phase independent, except mismatch and recombination repair. Cells containing damaged genomic DNA arrest at the G_1/S and G_2/M transitions, so as to gain time for repair and to avoid fixing mutations during replication and cell division. p53, which can be induced by DNA damage, plays a central role in: (i) arresting cells in G_1 through induction of GADD45, MDM2 and CIPI/WAF1, which in turn inhibit cyclin-dependent kinases required for the G_1/S transition; (ii) arresting cells in G_2 in an indirect but unknown way; (iii) induction of DNA repair; (iv) apoptosis by up-regulation of Bax and down-regulation of bcl-2 if cellular p53 levels are too high. However, p53 is not necessarily required for G_1 or G_2 arrest or apoptosis.

The expression levels of some repair genes fluctuate during the cell cycle (Stich, 1975; Mitra and Kaina, 1993) under the control of cell cycle-dependent transcription factors, such as E2F-1 (Dosanjh *et al.*, 1994). On the other hand, high level expression of some repair enzymes can delay growth in G_1 phase and stop the cells from progressing into S phase (Dosanjh *et al.*, 1994). These observations demonstrate the close relationship between DNA repair and cell cycle regulation. This is the most intricate factor for DNA repair, because cells in different stages of the cell cycle may react quite differently to DNA damage. Cell cycle block can also be induced by mutagens/aneugens which have non-DNA targets, e.g. spindle poisons. Casenghi *et al.* (1999) and Verdoodt *et al.* (1999) demonstrated p53-independent apoptosis and p53-dependent block of DNA re-replication, and thus arrest in G_1/S, following mitotic spindle inhibition by nocodazole in human cells.

The repair and apoptotic processes are therefore likely to influence both the expression of MNi *in vivo* during *in vivo* nuclear division and MNi expression *in vitro*. The expected division delay in cells with damaged DNA is therefore likely to cause MNBN cells to appear at a later time in culture than non-damaged cells. In their studies with the CBMN assay of X-irradiated human lymphocytes, Scott *et al.* (1998) showed that MNi frequency in BN cells increased steadily between 68 and 76 h, with a plateau level thereafter up to 96 h. These studies suggest that harvesting BN cells at a later time than the standard 72 h may better ensure that all DNA-damaged cells complete nuclear division before harvest. The appearance of the plateau in MNi expression in BN cells should be well researched for each specific set of culture conditions before launching into any study with the CBMN technique.

In vitro apoptosis and necrosis of damaged cells

Necrotic cells release their cytoplasmic content of toxic enzymes into the surrounding tissue, which usually causes a local inflammatory reaction. In contrast, apoptosis is a controlled form of cell death, where the integrity of the plasma membrane is preserved until late in the process, thus enabling packaging of disintegrating organelles into membrane-bound vesicles without leakage of intracellular components. Apoptotic cells or cellular fragments are eventually phagocytosed by neighbouring cells or macrophages without inducing an inflammatory reaction. Thus apoptosis is considered a means of removal of individual cells without producing tissue damage (Zakeri and Ahuja, 1997; Kolesnick and Krönke, 1998). The sequences of cytological events, the timing and the genotoxin-dependent pathways which characterize necrosis and apoptosis are summarized in Table I.

As far as biomonitoring is concerned, the genotoxic events which may induce apoptosis or necrosis *ex vivo*/*in vitro* may include DNA adducts, DNA breaks and/or protein adducts which accumulated during the *in vivo* exposure and/or to genotoxins present in the donor serum (if whole blood cultures are used for biomonitoring) and therefore in the culture medium. One may expect either that necrosis and apoptosis are triggered directly after the start of *in vitro* cultivation or that the lymphocyte requires stimulation/cycling to respond to necrotic/apoptotic stimuli; the latter is more probable, otherwise one may wonder why accumulated adducts did not induce cell death earlier *in vivo*, in resting G_0 cells. Considering an *in vitro* culture period of 72 (or 68) h after T lymphocyte stimulation with phytohaemagglutinin (PHA), as usually applied for the *in vitro* MN test, the first apoptotic and necrotic cells may therefore be expected between 15 min and 24 h in G_0 and G_1 cells, respectively, after cell cultivation. Even if apoptosis/necrosis is triggered only after 24 h, it is still achieved before mitosis. It is therefore clear that both apoptosis and necrosis can modify the number of cells which reach the first mitosis (on average 48 h after start of culture) and progress to the metaphase/anaphase transition to give rise to a BN cell in the presence of cytochalasin B. Recently, necrosis and apoptosis measurements have been integrated into the CBMN assay and scoring criteria for these end-points of toxicity were defined; the results from these studies showed that the main event

Table I. Comparison of apoptosis and necrosis

Apoptosis	Necrosis
Physiological impact	
Affects individual cells	Affects groups of contiguous cells
No inflammation/no tissue damage	Inflammatory response with tissue damage
Sequence of morphological features	
Shrinking of cytoplasm; condensation of nucleus	Loss of membrane integrity
Formation of apoptotic bodies with intact organelles	Swelling of cytoplasm and mitochondria by influx of water ions
No release of toxic enzymes	No vesicle formation; lysis of organelles and entire cell
Membrane integrity until late stage	Release of toxic enzymes into surrounding tissue
Biochemical characteristics	
Active process involving activation and enzymatic steps	Passive process (no energy required)
Activation of caspase cascade	Loss of regulation of ion homeostasis
Non-random mono- and oligonucleosomal length DNA fragmentation	Mainly random DNA fragmentation
Genotoxin-dependent pathways	
Induction	
Occurs under normal physiological conditions and can be triggered by specific stimuli such as DNA damaging agents, microtubule-targeted drugs, irradiation, activation of cell death receptors, lack of growth receptors	Occurs when cells are irreversibly damaged by extremes of temperature or pH, hypoxia, toxic concentrations of a variety of agents or induced membrane damage or physical trauma (manipulation)
Pathways	
Depending on the specific stimuli, the apoptotic process is triggered via a specific programmed pathway: membrane: Fas, TNF, ceramide, MEKK1, caspases; microtubules: Ras, MEKK1, Bax, caspases; DNA: Bax, caspases	No specific pathways involved depending on the type of damage; rather, a passive, 'accidental' process
Timing	
Depending on the type of induction, apoptosis can occur very early, e.g. via membrane or cytoskeleton after 15 min, or later, e.g. directly damaging DNA after 24 h	No clear correlation between type of damage and timing

induced by H_2O_2 in human lymphocytes exposed at G_0 was necrosis, which was at least 50 times more prevalent than apoptosis and 25 times more frequent than MNBN cells (Fenech *et al.*, 1999b).

Mitotic slippage

Spindle inhibitors are currently employed to effectively induce mitotic arrest, since they activate a specific checkpoint at the metaphase to anaphase transition, which monitors assembly of the mitotic spindle as well as complete chromosome alignment; in the presence of defects, the checkpoint blocks the completion of mitotic division (for reviews see Gorbsky, 1997; Hardwick, 1998). At least two classes of such agents are known (Johnson *et al.*, 1993): (i) inhibitors of microtubule polymerization, such as colchicine, carbendazim and nocodazole; (ii) microtubule dynamics stabilizers, exemplified by taxol. Both classes block or delay the metaphase to anaphase transition (Kirsch-Volders *et al.*, 1998), sustained by high levels of active maturation promoting factor (MPF) (Gorbsky, 1997), i.e. cyclin B1 associated with p34cdc2 kinase. Mitotic exit and progression to the following interphase require timely degradation of the cyclin B1 component (Norbury and Nurse, 1992). However, even in the absence of a functional mitotic spindle and consequent failure of chromatid migration to the poles, MPF can undergo spontaneous inactivation; this process, known as mitotic slippage, yields 4N cells. Elhajouji *et al.* (1998) analysed the frequencies of MNi in MONO cells in a control population and after *in vitro* exposure to aneugenic compounds. Since a clear increase in MNi in MONO cells was found only after exposure to aneugenic compounds and polyploidy/aneuploidy was found by means of chromosome-specific fluorescence *in situ* hybridisation (FISH) in these cells, the authors concluded that some lymphocytes with a deficient microtubule apparatus pass mitosis without chromatid segregation to daughter nuclei, obviously do not undergo cytokinesis and therefore remain MONO cells despite the presence of cytochalasin B. Exposure to spindle poisons may thus induce mitotic slippage and contribute to lowered frequencies of MNBN. Whether these observations can be extrapolated to other aneugens has not yet been demonstrated. Whether *in vivo* exposure to mitotic spindle inhibitors could cause mitotic slippage during *ex vivo* culture in the MN assay remains an unanswered question.

Does MN expression following in vitro culture reflect MN expression in vivo?

All assays of DNA damage require, to varying degrees, some treatment of cells prior to actual scoring of the end-point. For example, DNA adduct assays require DNA to be isolated from cells and this procedure itself may artefactually alter the frequency of adducts observed. Similarly, the conventional MN assay in lymphocytes requires that blood be diluted in culture medium, followed by treatment with lectin and exposure to culture conditions for 48–96 h. In the CBMN assay an additional treatment with cytochalasin B is required to accumulate BN cells. Ideally we would want to replicate the *in vivo* conditions and mimic the outcome of nuclear division *in vivo*. To do this would require a culture medium that is almost identical to the lymph node, spleen or bone marrow environment in terms of cell density, oxygen tension and chemical composition of tissue fluid. In fact, the culture medium most often used is usually adequate in terms of vitamin mixture but relatively low in serum factors that are known to be powerful antioxidants in blood (e.g. uric acid, albumin, etc.). It is therefore important to consider that the MN index observed following *in vitro* nuclear division may be influenced by culture conditions. Our studies to date have shown that differences in culture medium (i.e. RPMI 1640 or McCoy's 5A medium) did not influence the MN index (Fenech,

1998a), but a more appropriate comparison would have been to culture in a medium that is very similar to the composition of human plasma. In the study of Peace *et al.* (1999) it was shown, in an individual with an extraordinarily high frequency of MNi (120 MNi per 1000 BN cells), that this was due to an abnormally high rate of loss of chromosome 2. Interestingly they observed that the proportion of MN with a positive signal for the chromosome 2 centromere was the same for both MNi in MONO cells (38% of MN positive for the chromosome 2 centromere) and MNi in BN cells (44% of MN positive for the chromosome 2 centromere), which led them to conclude that the CBMN method accurately produces MNi with the same chromosomal content as those produced *in vivo*. More studies like this are needed to confirm this conclusion.

Factors that may cause a negative result in the CBMN assay in biomonitoring when exposure to specific genotoxins is evident

Aneugens

Comparison of MN frequencies in MONO and BN cells after *in vitro* treatment with aneugens acting through interaction with the spindle microtubules (nocodazole, mebendazole, colchicine and carbendazim) versus clastogens (methylmethane sulphonate and mitomycin C) showed that aneugens, but not clastogens, clearly induced an increase in MNi in both MONO and BN cells. In general the frequencies are higher in BN cells than in MONO cells, except for colchicine (Elhajouiji *et al.*, 1998). These data were interpreted as being the result of mitotic slippage. Scoring of BN cells alone for biomonitoring purposes would eventually lead to false negatives if the aneugen at low chronic levels of exposure shows a threshold type of dose–response effect (Elhajouji *et al.*, 1995, 1997); in this case, *in vitro* some cells exposed at a below threshold concentration would traverse the metaphase/anaphase transition without induction of MNi and those cells exposed above the threshold concentration would undergo mitotic slippage and become MNed MONO cells. Moreover, mitotic slippage might have been introduced *in vivo*, leading to both MNed MONO cells and polyploid MONO cells in freshly isolated lymphocytes which could be observed before nuclear division *in vitro*.

Chronic low level exposure and adaptive response

Adaptive responses are observed when cells become resistant to a high dose of a cytotoxic agent after low dose exposure to that agent or another genotoxic agent. Adaptation describes resistance to a challenging dose of the same agent as used for the initial treatment. Cross-adaptation occurs when exposure to low doses of radiomimetic chemicals, alkylating agents, cross-linking agents or ionizing radiation leads to a decrease in the cell's sentivity to the same agent or to any of the others. These responses have been attributed to the induction of a repair mechanism by the low dose exposure (for a review see Wolff, 1998). Boothman *et al.* (1996) hypothesized that only certain human cells can adapt to ionizing radiation by progressing to a point later in G_1 where DNA repair processes and radioresistance can be induced and proposed that a protein complex composed of cyclin D1, PCNA and, possibly, cyclin A may play a role in cell cycle regulation and DNA repair, which determines the adaptive response in human cells. The induction of an adaptive response in human lymphocytes from workers occupationally exposed to mutagens has been observed for several genotoxic end-points, including chromosome aberrations (Barquinero *et al.*, 1995) and MNi formation (Gourabi and Mozdarani, 1998), and seems to depend on the nature of the challenge, the total dose of the pre-treatment and the dose rate for ionizing radiation (Shadley and Wiencke, 1989).

A question which has not yet been addressed is whether chronic low level exposure to toxins may induce an adaptive response related to the induction of an increase in apoptosis sensitivity or a more extended cell cycle delay to enable repair to proceed appropriately. This would mean that adapted cells that have significant DNA damage may, during culture, have a greater propensity to undergo apoptosis or delay nuclear division. This may prevent detection of exposure effects by MNi in BN cells because the damaged cells would either preferentially undergo apoptosis or, if they proceeded with nuclear division, might appear as BN cells at a considerably later time than the typical harvest time. The comprehensive CBMN assay we propose should overcome these problems and also verify whether sensitivity to apoptosis is increased by chronic exposure to genotoxins.

Some exposures may produce mainly adducts and few strand breaks: an ARA-C assay may be needed to convert excision repaired sites to micronuclei

In previous studies we have shown that lymphocytes exposed in G_0 to genotoxins that mainly induce DNA adducts and not strand breaks do not efficiently induce MN formation in once-divided cells. We argued that if exposure to such an agent occurred (*in vivo* or *in vitro*) then it should be possible to convert excision repair sites to single-stranded DNA breaks by inhibiting the gap filling step using cytosine arabinoside (ARA-C protocol) during G_1 (i.e. the first 16 h post-PHA stimulation). With this approach we showed that the sensitivity of the CBMN assay was increased at least 10-fold following exposure to UV radiation or methyl nitrosourea (Fenech and Neville, 1992). This implies that the CBMN assay would only be able to detect exposure to such agents if the ARA-C protocol was used. However, it is also known that such agents are inducers of chromosome breaks and MNi when cells are exposed during S phase (Galloway *et al.*, 1998; Keshava *et al.*, 1998). This implies that MNi may be induced *in vivo* in dividing cell populations exposed to these types of agents leading to MNed G_0 lymphocytes. However, this damage may not be detectable in BN cells in the CBMN assay because: (i) the MNed MONO cells may not divide; (ii) G_0 lymphocytes with adducts would only express these lesions as MNi in BN cells if the ARA-C protocol is used.

Sensitivity of MONO cells and BN cells in relation to the genotoxic mechanism

The assessment of MN in BN cells remains an important and sensitive method for biomonitoring of exposure to ionizing radiation (Fenech *et al.*, 1990; Chang *et al.*, 1997) and clastogenic chemicals (e.g. nitrous oxide, complex mixtures of polycyclic aromatic hydrocarbons and antineoplastic drugs) (Osanto *et al.*, 1991; Chang *et al.*, 1996; Somorovska *et al.*, 1999) that induce MNi during *ex vivo* culture when lymphocytes are exposed *in vivo* in G_0. It is evident that it may be less useful with agents that are not clastogenic when exposure occurs in G_0 and/or mainly induce adducts but not strand breaks in DNA (Fenech and Neville, 1992) or agents that are spindle or centromere/kinetochore poisons. In these cases the ARA-C protocol (for agents that are non-clastogenic when exposure occurs in G_0 and that induce excision repairable

DNA adducts) or scoring of MNi in MONO cells (for agents that are clastogenic or induce chormosome loss in G_1/S/G_2 cells *in vivo*) may be more successful.

One of the key factors in the use of MN in MONO cells is expected to be the timing of exposure relative to the time of blood collection. The probability that MN would be observed in MONO cells may depend on the exposure period. The probability of observing a MONO cell with induced MN will also depend on the proliferation rate of the lymphocyte subset to which it belongs and the half-life of that subset of lymphocytes.

With regard to T lymphocytes, the kinetics of cell division and the lifespan in peripheral blood varies depending on whether the T lymphocyte is a long-lived 'memory' cell or whether it is a short-lived uncommitted 'naive' cell (half-life of a few days). It is possible to distinguish between and isolate 'memory' and 'naive' cells using antibodies to surface markers ($CD45RO^+$ and $CD45RA^+$, respectively) (Rosenmann *et al.*, 1998; Rufer *et al.*, 1998) and score MN specifically in these populations. Telomere length analysis suggests that 'naive' cells have undergone fewer cell divisions than 'memory' cells (Rufer *et al.*, 1998). At this time we have insufficient information on the kinetics of MN expression in these subsets of T lymphocytes *in vivo* but one might expect cells with a higher turnover rate to be more responsive to recent clastogen exposure in terms of *in vivo* expression of MNi in MONO cells.

Proposed new methodology for a more comprehensive CBMN assay

It is evident that a more comprehensive assessment of DNA damage in lymphocytes could be achieved if both MN in uncultured freshly isolated lymphocytes and MN expressed in cultured lymphocytes were scored. It should also be stated that while scoring of MN in lymphocytes from blood smears provides a practical and inexpensive method for monitoring genetic damage in chronically exposed populations, the number of cells that can be scored is limited by the proportion of lymphocytes with a large cytoplasmic/nucleoplasmic ratio. However, it has been reported recently that 2000 scorable lymphocytes can be prepared from two drops of whole blood following treatment with methylcellulose solution (Xue *et al.*, 1992). An alternative procedure that would greatly facilitate the scoring of MNi in non-divided lymphocytes is to set up whole blood or isolated lymphocyte cultures which are stimulated to enter the cell division cycle by PHA. By harvesting cells before they enter mitosis one can allow the cytoplasm to expand and enable MN to be readily observed mainly in the responding T lymphocytes; this same culture can then be used for cytokinesis blockage and harvesting of BN cells at a later time point (72–96 h post-PHA stimulation). We recommend harvesting MONO cells at 24 h post-PHA stimulation, as there can be no doubt at this time point that MN within such a cell are a result of *in vivo* rather than *ex vivo* division and, furthermore, MN scored in activated MONO cells at 24 h are more directly comparable with MN in BN cells, as one can be almost certain that the activated MONO cells are from similar lymphocyte subtypes as the BN cells. We also advise that the 24 h post-PHA time point may be the right time to count apoptotic/necrotic cells, as the bulk of stimulated cells are likely to have committed themselves to the DNA synthesis route or the apoptosis/necrosis pathways by this stage. However, one cannot exclude that late dividing

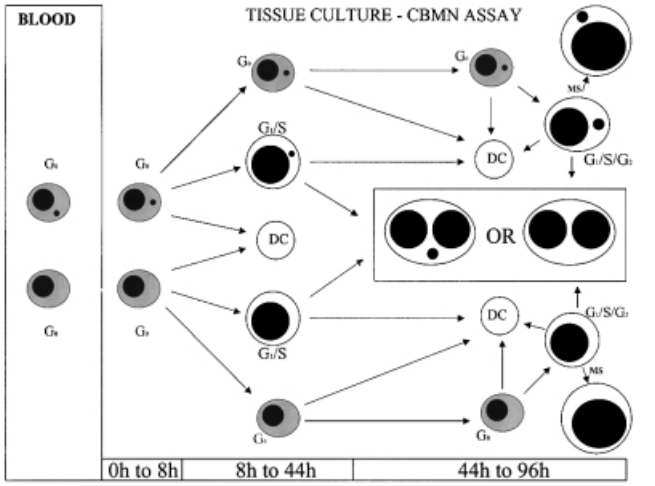

Fig. 2. MN expression (*in vivo* and *ex vivo*) and fate of MNed cells in the CBMN assay. DC, dead cell due to necrosis or apoptosis; MS, mitotic slippage. Cells with grey cytoplasm are non-dividing cells. Cells with white cytoplasm represent cells that are actively undergoing nuclear division at the designated cell cycle stage.

cells may opt for necrosis or apoptosis at a later culture time. In fact, one can observe numerous necrotic and apoptotic cells at 72 h post-PHA in typical lymphocyte cultures (Fenech *et al.*, 1999b).

A scheme describing the various events that may occur as lymphocytes (which may or may not contain a MN before PHA stimulation) progress through nuclear division in cytokinesis block cultures is illustrated in Figure 2. Figure 3 shows two alternative cell harvesting time and cell scoring protocols.

In the current CBMN assay protocol all measures of DNA damage are restricted to MN measurements in BN cells. This narrow approach does not take into account that non-dividing cells may include pre-MNed cells and that some of the damaged cells may opt to undergo apoptosis or necrosis instead of dividing. We have therefore proposed that apoptosis (Kirsch-Volders *et al.*, 1997) and necrosis (Fenech *et al.*, 1999b) should be integrated into the assay as well as measures of frequency of MNed MONO cells (Fenech *et al.*, 1997; Elhajouji *et al.*, 1998).

It is important to note that the probability of a MNed lymphocyte completing nuclear division *in vitro* after PHA stimulation may be much less than that of a non-MNed lymphocyte. Recent studies by Sablina *et al.* (1998) suggest a p53-mediated cell cycle checkpoint preventing proliferation of MNed cells and that abolition of p53 function allowed MNed cells to complete nuclear division.

It has also become evident that apoptosis can be inhibited by overexpression of bcl-2 as well as by survivin, which acts, independently of bcl-2, in the G_2/M phase of the cell cycle (Li and Altieri, 1999; Fussenegger *et al.*, 2000). The addition of apoptosis inhibitors, such as survivin, to the cell culture during the CBMN assay may enable most of the 'viable' DNA-damaged cells to be expressed as MNed BN cells rather than defaulting into apoptosis.

From the above considerations and published results (Fenech *et al.*, 1999b) it is clear that by scoring multiple cytological events in the CBMN assay, one can achieve a much more complete and correct assessment of toxicity.

Conclusions

A more comprehensive assessment of DNA damage and genome mutations may be achieved if the CBMN assay

A

Proposed comprehensive CBMN assay for biomonitoring
Method #1

0h	Add PHA
44h	Add Cytochalasin B
72h	Harvest cells and score * MN in transformed MONO cells * MN in BN cells * Necrotic and apoptotic cells

B

Proposed comprehensive CBMN assay for biomonitoring
Method #2

0h	Add PHA
24h	Harvest cells and score * MN in transformed MONO cells * Necrotic & apoptotic cells
44h	Add Cytochalasin B
72h	Harvest cells and score * MN in BN cells

Fig. 3.

includes measures of: (i) MNi in MONO cells; (ii) MNi in BN cells; (iii) apoptotic cells; (iv) necrotic cells. It is probable that the 24 h post-PHA time point may be the optimal time to assess the frequency of MNi in MONO cells, apoptotic cells and necrotic cells. It is also practical to include these measures when scoring MNi in BN cells after cytokinesis block. It is clear that much research is needed in defining the effect of *in vivo* cell division kinetics on MN expression in MONO cells from different lymphocyte subsets. We look forward to future studies with this comprehensive approach.

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