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The in vitro micronucleus technique

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Abstract

The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably. Because micronuclei can only be expressed in cells that complete nuclear division a special method was developed that identifies such cells by their binucleate appearance when blocked from performing cytokinesis by cytochalasin-B (Cyt-B), a microfilament-assembly inhibitor. The cytokinesis-block micronucleus (CBMN) assay allows better precision because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions. The method is now applied to various cell types for population monitoring of genetic damage, screening of chemicals for genotoxic potential and for specific purposes such as the prediction of the radiosensitivity of tumours and the inter-individual variation in radiosensitivity. In its current basic form the CBMN assay can provide, using simple morphological criteria, the following measures of genotoxicity and cytotoxicity: chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), cell division inhibition, necrosis and apoptosis. The cytosine-arabinoside modification of the CBMN assay allows for measurement of excision repairable lesions. The use of molecular probes enables chromosome loss to be distinguished from chromosome breakage and importantly non-disjunction in non-micronucleated binucleated cells can be efficiently measured. The in vitro CBMN technique, therefore, provides multiple and complementary measures of genotoxicity and cytotoxicity which can be achieved with relative ease within one system. The basic principles and methods (including detailed scoring criteria for all the genotoxicity and cytotoxicity end-points) of the CBMN assay are described and areas for future development identified. © 2000 Published by Elsevier Science B.V.

Keywords: DNA damage; Chromosome; Genetic toxicology

Abbreviations: ARA=cytosine arabinoside; BN=binucleated; CBMN assay=cytokinesis-block micronucleus assay; Cyt-B=cytochalasin-B; DC=deoxycytidine; IL-2=interleukin-2; MN=micronucleus; MNi=micronuclei; PHA=phytohaemagglutinin

1. Introduction

The observation that chromosome damage can be caused by exposure to ionising radiation or carcinogenic chemicals was among the first reliable evidence that physical and chemical agents can cause major alterations to the genetic material of eukaryotic cells [1]. Although our understanding of chromosome structure is incomplete, evidence suggests that chromosome abnormalities are a direct consequence and manifestation of damage at the DNA level — for example, chromosome breaks may result from unrepaired double strand breaks in DNA and chromosome rearrangements may result from misrepair of strand breaks in DNA [2]. It is also recognised that chromosome loss and malsegregation of chromosomes (non-disjunction) are an important event in cancer and ageing and that they are

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probably caused by defects in the spindle, centromere or as a consequence of undercondensation of chromosome structure before metaphase [3–5].

In the classical cytogenetic techniques, chromosomes are studied directly by observing and counting aberrations in metaphases [6]. This approach provides the most detailed analysis, but the complexity and laboriousness of enumerating aberrations in metaphase and the confounding effect of artefactual loss of chromosomes from metaphase preparations has stimulated the development of a simpler system of measuring chromosome damage.

It was proposed independently by Schmid [7] and Heddle [8] that an alternative and simpler approach to assess chromosome damage in vivo was to measure micronuclei (MNi), also known as Howell–Jolly bodies to haematologists, in dividing cell populations such as the bone-marrow. The micronucleus assay in bone-marrow and peripheral blood erythrocytes is now one of the best established in vivo cytogenetic assays in the field of genetic toxicology, however, it is not a technique that is applicable to other cell populations in vivo or in vitro and methods have since been developed for measuring MNi in a variety of nucleated cells in vitro.

MNi are expressed in dividing cells that either contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to travel to the spindle poles during mitosis. At telophase, a nuclear envelope forms around the lagging chromosomes and fragments, which then uncoil and gradually assume the morphology of an interphase nucleus with the exception that they are smaller than the main nuclei in the cell, hence the term “micronucleus” (Fig. 1). MNi, therefore, provide a convenient and reliable index of both chromosome breakage and chromosome loss. Because MNi are expressed in cells that have completed nuclear division they are ideally scored in the binucleated stage of the cell cycle [9,10]. Occasionally nucleoplasmic bridges between nuclei in a binucleated cell are observed. These are probably dicentric chromosomes in which the two centromeres were pulled to opposite poles of the cell and the DNA in the resulting bridge covered by nuclear membrane (Fig. 1). Thus, nucleoplasmic bridges in binucleated cells provide an additional and complementary measure of chromosome rearrangement,

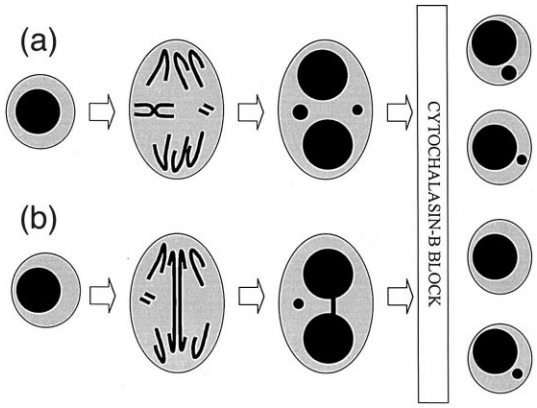

Fig. 1. (a) The origin of micronuclei from lagging whole chromosomes and acentric chromosome fragments at anaphase. (b) The formation of a nucleoplasmic bridge from a dicentric chromosome in which the centromeres are pulled to opposite poles of the cell; the formation of a micronucleus from the accompanying acentric chromosome fragment is also illustrated. The critical role of cytochalasin-B in blocking dividing cells at the binucleate stage is also indicated in this diagram. The example shown is for a hypothetical cell with two pairs of chromosomes only.

which can be scored together with the micronucleus count.

It is evident from the above that MNi can only be expressed in dividing eukaryotic cells. In other words, the assay cannot be used efficiently or quantitatively in non-dividing cell populations or in dividing cell populations in which the kinetics of cell division is not well understood or controlled. Consequently, there was a need to develop a method that could distinguish between cells that are not dividing and cells that are undergoing mitosis within a cell population. Furthermore, because of the uncertainty of the fate of MNi following more than one nuclear division it is important to identify cells that have completed one nuclear division only. These requirements are also necessary because cells divide at different rates in vivo and in vitro depending on the various physiological, genetic and micronutrient conditions.

Several methods have been proposed based on stathmokinetic, flow cytometric and DNA labelling approaches but the method that has found most favour due to its simplicity and lack of uncertainty regarding its effect on base-line genetic damage is the cytokinesis-block micronucleus (CBMN) assay [9–11].

In the CBMN assay, cells that have completed one nuclear division are blocked from performing cytokinesis using cytochalasin-B (Cyt-B) and are consequently readily identified by their binucleated appearance (Fig. 1). Cyt-B is an inhibitor of actin polymerisation required for the formation of the microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis [12]. The use of Cyt-B enables the accumulation of virtually all dividing cells at the binucleate stage in dividing cell populations regardless of their degree of synchrony and the proportion of dividing cells. MNi are then scored in binucleated cells only, which enables reliable comparisons of chromosome damage between cell populations that may differ in their cell division kinetics. The method was initially developed for use with cultured human lymphocytes [9,10], but has now been adapted to various cell types such as solid tumour and bone-marrow cells [13,14]. Furthermore, new developments have also occurred that allow (a) MNi originating from whole chromosomes to be distinguished from MNi originating from chromosome fragments [15–20], (b) the conversion of excision-repaired sites to MNi within one cell division [21], (c) the use of molecular probes to identify non-disjunction events in binucleated cells [22–24] and (d) the integration of necrotic and apoptotic cells within the CBMN assay [25,26].

It has recently been proposed that the micronucleus assay be used instead of metaphase analysis for genotoxicity testing of new chemicals. A recent special issue of Mutation Research has been dedicated to this topic [27]. The current methodologies and data for the in vitro micronucleus test were reviewed at the Washington International Workshop on Genotoxicity Test Procedures which was held in 1999 [28].

The standard CBMN assay and its various modifications are described in detail in the next sections. The methods described are mainly applicable to cultured human lymphocytes, however, modifications of the assay for application to other cell types are included.

1.1. Standard cytokinesis-block micronucleus assay for isolated human lymphocytes

In this technique MNi are scored only in those cells that have completed one nuclear division following phytohaemagglutinin (PHA) stimulation. These cells are recognised by their binucleated appearance after they are blocked from performing cytokinesis by Cyt-B which should be added before the first mitotic wave. Optimal culture conditions should yield 35–60% or more binucleates as a proportion of viable cells (i.e., all cells excluding necrotic and apoptotic cells) at 72 h after PHA stimulation. All equipment should have biosafety features to protect the operator and solutions used in this procedure should be filter sterilised.

1.1.1. Lymphocyte isolation, cell culture and cell harvesting

(1) Fresh blood is collected by venepuncture in tubes with heparin as anticoagulant and stored at 22°C for less than 4 h prior to lymphocyte isolation.

(2) The blood is then diluted 1:1 with isotonic (0.85%) sterile saline and gently inverted to mix.

(3) The diluted blood is overlaid gently on Ficoll Paque (Pharmacia) density gradients using a ratio of approximately 1:3 (e.g., 2 ml Ficoll Paque to 6 ml of diluted blood), being very careful not to disturb the interface.

(4) The gradient is then spun in a centrifuge at $400\times g$ for 25–40 min at 22°C after carefully balancing the tubes.

(5) The lymphocyte layer at the interface of Ficoll Paque and diluted plasma is collected with a sterile plugged pasteur pipette and added to 3–5 times volume of Hanks balanced salt solution (HBSS) at 22°C. The resulting cell suspension is centrifuged at $280–400\times g$ for 5–10 min depending on the volume.

(6) The supernatant is discarded, the cells resuspended in 2–5 times volume HBSS and centrifuged at $180–400\times g$ for 5 min depending on the volume.

(7) The supernatant is discarded and the cells resuspended in 1 ml RPMI 1640 culture medium.

(8) Cell concentration is then measured using a Coulter Counter or haemocytometer and the concentration adjusted by the percentage of viable cells measured using trypan blue exclusion assay.

(9) The cells are resuspended in RPMI 1640 medium containing 10–15% heat inactivated foetal calf serum at $0.5–1.0\times10^6$ cells/ml and cultured in 0.75–1.0 ml volume in round–bottom tissue culture tubes (10 mm width).

(10) Lymphocytes are then stimulated to divide by adding phytohaemagglutinin (PHA) (Glaxo Wellcome

HA15) to each culture tube at 10 μl/ml (from a stock solution in H_2O of 2.25 mg/ml) and incubated at 37°C with loose lids in a humidified atmosphere containing 5% CO_2. The concentration of PHA used has to be optimised depending on the purity and source of the reagent to ensure maximum number of binucleated cells after Cyt-B block.

(11) Forty-four hours after PHA stimulation, 4.5 μg Cyt-B is added to each milliliter of culture [USE GLOVES AND FUME HOOD]: a 100 μl aliquot of Cyt-B stock solution in DMSO (600 μg/ml) is thawed, 900 μl culture medium added and mixed. Seventy-five microliters of the mixture is added to each 1 ml of culture to give a final concentration of 4.5 μg Cyt-B/ml (other laboratories have successfully used 6.0 μg Cyt-B/ml in their cultures). Culture tubes are then re-incubated with loose lids.

(12) Twenty-eight hours after adding Cyt-B, cells are harvested by cytocentrifugation (Shandon Elliot). One hundred microliters of the culture medium is removed without disturbing the cells and then cells are gently resuspended in their tubes. 100–120 μl of cell suspension is transferred to cytocentrifuge cups (Shandon Elliot) and centrifuged to produce 2 spots per slide [Set the cytocentrifuge as follows — time: 5 min, speed: 600 rpm]. Slides are removed from the cytocentrifuge and allowed to air dry for 10–12 min *only* and then fixed for 10 min in absolute methanol.

(13) The cells can be stained using a variety of techniques that can clearly identify nuclear and cytoplasmic boundaries. In our experience, the use of "Diff Quik" (Lab-Aids, Australia), a commercial ready-to-use product, provides rapid and optimal results.

(14) After staining, the slides are air-dried and coverslips placed over the cells using Depex (DPX) mounting medium. This procedure is carried out in the fume hood and the slides are left to set in the fume hood and then stored indefinitely until required.

Important note: Duplicate cultures of control or genotoxin-treated cells should be set up and slides from each culture should be prepared. This is essential to obtain a measure of experimental variation, i.e., coefficient of variation, which should be quoted with each set of duplicate cultures. This experimental design is summarised in Fig. 2.

For fluorescence microscopy staining with acridine orange (40 μg/ml in Sorensen's phosphate buffer pH 6.9) is recommended. If a cytocentrifuge is not available, slides can be prepared using the procedure, described below, for whole blood cultures.

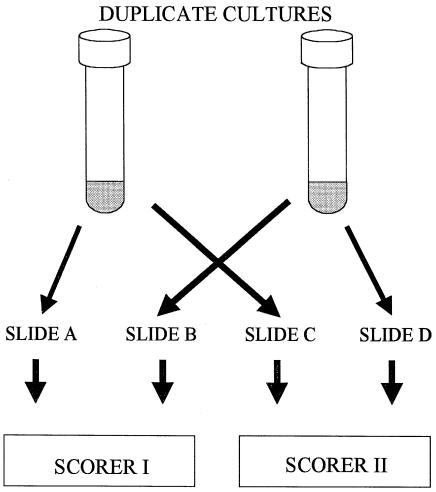

Fig. 2. An optimal sampling schedule for the in vitro micronucleus assay that enables an estimation of experimental variation (results for A+C versus B+D) as well as the effect of scorer bias (results for A+B versus C+D).

1.2. Examination of slides and assessment of MN frequency

Slides are best examined at 1000× magnification using a light or fluorescence microscope. Slides should be coded before analysis so that the scorer is not aware of the identity of the slide. A score should be obtained for slides from each duplicate culture. The number of cells scored should be determined depending on the level of change in the MN index that the experiment is intended to detect and the expected standard deviation of the estimate. For each slide the following information should be obtained:

1. The number of micronuclei (MNi) in at least 1000 binucleate [BN] cells should be scored and the frequency of MNi per 1000 BN cells calculated. The criteria for scoring MNi in BN cells are detailed below.
2. The distribution of BN cells with zero, one or more MNi; the number of MNi in a single binucleated cell normally ranges from 0 to 3 in lymphocytes of healthy individuals but can be greater than 3

on occasion depending on genotoxin exposure and age.
3. The frequency of micronucleated BN cells in at least 1000 BN cells.
4. The frequency of nucleoplasmic bridges in 1000 BN cells. Scoring criteria for nucleoplasmic bridges are described below.
5. The proportion of mononucleated, binucleated, tri-nucleated and tetra-nucleated cells per 500 cells scored. From this information, the Nuclear Division Index (explained below) can be derived.
6. The number of dead or dying cells due to apoptosis or necrosis per 500 cells may also be scored on the same slide (scoring criteria for these cells are detailed below) while scoring the frequency of viable mono-, bi- and multi-nucleated cells.

It is important to note that it is best to skip scoring a cell if one is uncertain on how to classify it. The basic elements of a typical score sheet are listed in Table 1.

1.3. Criteria for selecting binucleated cells which can be scored for micronucleus frequency

The cytokinesis-blocked cells that may be scored for MN frequency should have the following characteristics:

(a) The cells should be binucleated;

Table 1
Information that should be included on a score sheet for the cytokinesis-block micronucleus assay[a]

1. Code Number of each slide
2. Number of BN cells scored
3. The distribution of BN cells with 0, 1, 2, 3, or more MNi in at least 1000 BN cells.
4. Total number of MNi in BN cells.
5. The frequency of MNi in 1000 BN cells
6. The frequency of micronucleated BN cells in 1000 BN cells
7. Proportion of BN cells with nucleoplasmic bridges
8. The proportion of mono-, bi-, tri- and tetranucleated cells in 500 viable cells
9. The frequency of BN cells in a total of 500 viable cells
10. The nuclear division index
11. The proportion of cells that are undergoing apoptosis or necrosis in 500 cells
12. The nuclear division cytotoxicity index
13 Coefficient of variation for duplicate estimates of above parameters

[a] [BN=binucleate; MNi=micronuclei].

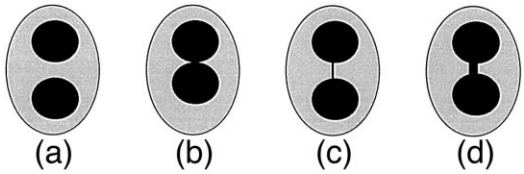

Fig. 3. Criteria for choosing binucleate cells in the cytokinesis-block micronucleus assay. (a) ideal binucleate cell; (b) binucleate cell with touching nuclei; (c) binucleate cell with narrow nucleoplasmic bridge between nuclei; (d) binucleate cell with relatively wide nucleoplasmic bridge. Cells with two overlapping nuclei may be considered suitable to score as binucleated cells if the nuclear boundaries are distinguishable. Occasionally binucleated cells with more than one nucleoplasmic bridge are observed.

(b) The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary;

(c) The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity;

(d) The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than 1/4th of the nuclear diameter.

(e) The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.

(f) The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

Examples of the type of binucleated cells that may or may not be scored are illustrated diagrammatically in Fig. 3. The cell types that should not be scored for micronucleus frequency include mono-, tri-, quadr- and multi-nucleated cells, and cells that are necrotic or apoptotic (illustrated in Fig. 4).

1.4. Criteria for scoring micronuclei

MNi are morphologically identical to but smaller than nuclei. They also have the following characteristics:

(a) The diameter of MNi in human lymphocytes usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.

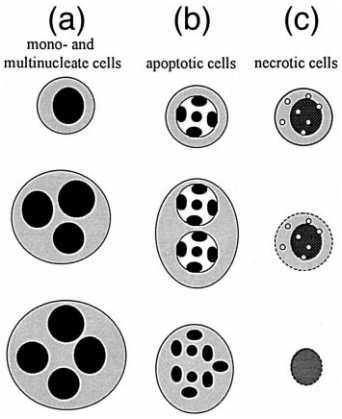

Fig. 4. The various types of cells that may be observed in the in vitro cytokinesis-block micronucleus assay excluding binucleated cells. These cell types shown should not be scored for MN frequency: (a) viable mono-, tri- and quadrinuclear cells; (b) mono- and binucleated cells at early stage of apoptosis when chromatin condensation has occurred but nuclear membrane has not disintegrated and late stage apoptotic cells with intact cytoplasm, no nucleus and apoptotic chromatin bodies within the cytoplasm; (c) cells at the various stages of necrosis including early stages showing vacuolisation, disintegration of cytoplasmic membrane and loss of cytoplasm with an intact nucleus and late stages in which cytoplasm is partially or completely lost and nuclear membrane is visibly damaged and nuclear material is commencing to leak from the remnant nucleus.

(b) MNi are non-refractile and they can therefore be readily distinguished from artefact such as staining particles;

(c) MNi are not linked or connected to the main nuclei;

(d) MNi may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary;

(e) MNi usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

Examples of typical MNi that meet the criteria set above are shown in Fig. 5. Examples of cellular structures that resemble MNi but should not be classified as MNi originating from chromosome breakage or loss are illustrated in Fig. 6. Induction of gene amplification may lead to extrusion of amplified genes into nuclear buds (e.g., Fig. 6c and d) during S phase that are eventually detached from the nucleus to form a micronucleus (Shimizu et al., 1998); it may be necessary

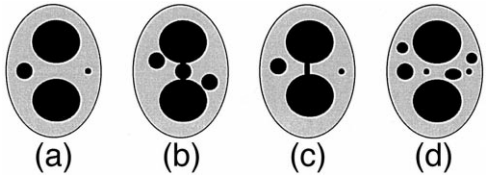

Fig. 5. Typical appearance and relative size of micronuclei in binucleated cells. (a) Cell with two micronuclei one with 1/3rd and the other 1/9th the diameter of one of the main nuclei within the cell. (b) Micronuclei touching but not overlapping the main nuclei. (c) A binucleated cell with nucleoplasmic bridge between main nuclei and two micronuclei. (d) A binucleated cell with six micronuclei of various sizes; this type of cell is rarely seen.

to quantify the frequency of nuclei with nuclear bud formation if gene amplification is suspected.

1.5. Criteria for scoring nucleoplasmic bridges

Nucleoplasmic bridges are sometimes observed in binucleated cells following exposure to clastogens. They are a continuous link between the nuclei in a binucleated cell and are thought to be due to dicentric chromosomes in which the centromeres were pulled to opposite poles during anaphase. The width of a nucleoplasmic bridge may vary considerably but usually does not exceed 1/4th of the diameter of the nuclei within the cell. The nucleoplasmic bridge should have the same staining characteristics of the main nuclei. On very rare occasions, more than one nucleoplasmic

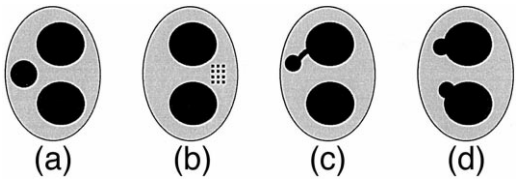

Fig. 6. Occasionally binucleated cells (or cells that resemble binucleated cells) may contain structures that resemble micronuclei but should not be scored as micronuclei originating from chromosome loss or chromosome breakage. These situations include (a) a trinucleated cell in which one of the nuclei is relatively small but has a diameter greater than 1/3 the diameter of the other nuclei; (b) dense stippling in a specific region of the cytoplasm; (c) extruded nuclear material that appears like a micronucleus with a narrow nucleoplasmic connection to the main nucleus and (d) nuclear blebs that have an obvious nucleoplasmic connection with the main nucleus.

bridge may be observed within one binucleated cell. A binucleated cell with a nucleoplasmic bridge often contains one or more micronuclei. Examples of binucleated cells with nucleoplasmic bridges are illustrated in Figs. 1 and 5.

1.6. Criteria for scoring apoptotic and necrotic cells

Fig. 7 describes the various pathways and events that may be expected to occur in cultured lymphocytes exposed to a toxic agent. Cytogenetic genotoxicity assays that require hypotonic treatment for the preparation of interphase cells (for whole blood micronucleus assay) or metaphase plates for chromosome analysis are not usable for cytotoxicity assays because hypotonic treatment may destroy necrotic cells and apoptotic cells making them unavailable for assay. Inclusion of necrosis and apoptosis is important for the accurate description of mechanism of action and measurement of cellular sensitivity to a chemical or radiation. Isolated lymphocyte culture assay or culture of cell lines does not require hypotonic treatment of cells for slide preparation, thus making it possible to preserve the morphology of both necrotic and apoptotic cells. The use of Cyt-B, should make it easier to score apoptotic cells because it is expected to inhibit the disintegration of apoptotic cells into smaller apoptotic bodies. The latter process requires microfilament assembly [29], which is readily inhibited by Cyt-B [12].

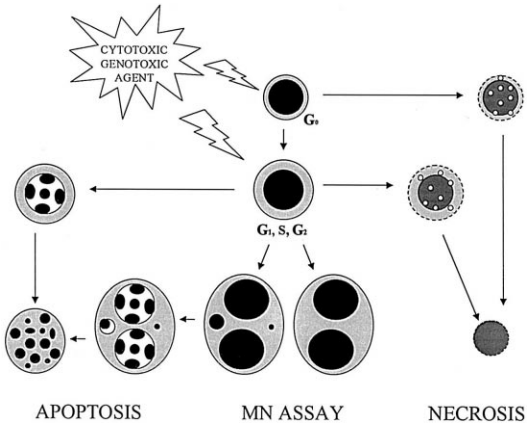

Fig. 7. The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents.

The following guidelines for scoring necrotic and apoptotic cells are recommended: (a) cells showing chromatin condensation with intact cytoplasmic and nuclear boundaries or cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane are classified as apoptotic; (b) cells exhibiting a pale cytoplasm with numerous vacuoles and damaged cytoplasmic membrane with a fairly intact nucleus or cells exhibiting loss of cytoplasm and damaged/irregular nuclear membrane with a partially intact nuclear structure are classified as necrotic. These criteria and results for these measures with hydrogen peroxide have been recently reported elsewhere [26].

Figs. 4 and 7 illustrate typical examples of necrotic and apoptotic cells.

1.7. Nuclear division index (NDI) and nuclear division cytotoxicity index (NDCI)

NDI is often calculated according to the method of Eastmond and Tucker [30]. Five hundred viable cells are scored to determine the frequency of cells with 1, 2, 3 or 4 nuclei and calculate the NDI using the formula:

$$\mathrm{NDI} = (\mathrm{M1} + 2(\mathrm{M2}) + 3(\mathrm{M3}) + 4(\mathrm{M4}))/N,$$

where M1–M4 represent the number of cells with one to four nuclei and N is the total number of viable cells scored. The NDI and the proportion of binucleated cells are useful parameters for comparing the mitogenic response of lymphocytes and cytostatic effects of agents examined in the assay.

A more accurate assessment of nuclear division status is obtained if necrotic and apoptotic cells are included in the total number of cells scored because at higher toxic doses of chemicals tested one can expect a very large proportion of cells becoming non-viable. It is therefore important to note that both binucleate ratio and the NDI are overestimated if necrotic and apoptotic cells are not included when scoring cells. A more accurate estimate of nuclear division status and cell division kinetics can be obtained using the following modified equation which takes account of viable as well as necrotic and apoptotic cells:

$$\text{NDCI} = (\text{Ap} + \text{Nec} + \text{M1} + 2(\text{M2}) + 3(\text{M3}) + 4(\text{M4}))/N^*,$$

where NDCI = nuclear division cytotoxicity index, Ap = number of apoptotic cells, Nec = number of necrotic cells, M1–M4 = number of viable cells with 1–4 nuclei and N^* = total number of cells scored (viable and non-viable).

1.8. Measurement of excision-repaired DNA lesions in G_0/G_1 human lymphocytes using the cytosine arabinoside micronucleus assay in human lymphocytes

After assessing the MN response in human G_0 lymphocytes following exposure to a variety of genotoxins it became evident that the extent of micronucleus formation in relation to cytotoxicity was low for chemicals and ultraviolet radiation which mainly induce base-lesions and adducts on DNA rather than strand breakage or spindle damage [21]. We hypothesised that this was due to either efficient repair of the lesions or that such sites, if left unrepaired, do not convert to a double stranded break in DNA following one round of DNA synthesis. Furthermore, we reasoned that inhibition of excision repair by cytosine arabinoside (ARA) would result in the conversion of such base lesions to a single stranded break which would become a double stranded break following DNA synthesis leading to the production of an acentric fragment which would then be expressed as a MN within one division cycle [21,31]. Using this concept (illustrated in Fig. 8) we showed that addition of ARA during the first 16 h of lymphocyte culture (i.e., before DNA synthesis) did result in a dramatic increase (10-fold or greater) in the MN dose–response following UV or MNU treatment. However, the ARA-induced increase following X-ray exposure was only 1.8-fold as would be expected from the proportion of DNA adducts or base lesions relative to the induction of DNA strand breaks. This method has since been used to identify pesticides that induce excision repair and to distinguish between genotoxic agents that do or do not induce excision repair [32]. The ARA protocol is an important adjunct to the basic CBMN assay and should be attempted particularly if strong cytotoxic effects are observed in conjunction with weak MN induction. Precise measurement of excision-repaired DNA lesions using the ARA method

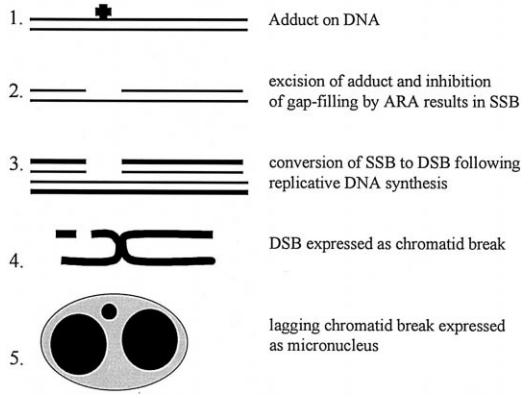

Fig. 8. A schematic diagram explaining the mechanism for the conversion by ARA of an excision-repairable DNA lesion to a micronucleus within one division cycle.

is only possible using the CBMN assay because (a) the conversion of excision-repaired DNA lesions to MN occurs only in cells that have completed nuclear division and (b) the addition of ARA may also result in significantly altered cell division kinetics which could confound results in MN assays without Cyt-B.

ARA inhibition of DNA polymerase may cause DNA strand breaks in cells undergoing replicative DNA synthesis. Therefore, it is only possible to use this method in PHA-stimulated G_0 lymphocytes with ARA exposure occurring during the G_1 phase and prior to S-phase, because excision repair is activated during G_1. In practice, this means that cells are cultured in the presence of ARA during the first 16–20 h after PHA stimulation, following which the cells are washed to remove ARA and incubated in culture medium containing deoxycyidine to reverse ARA inhibition of DNA polymerase; after these steps the standard CBMN protocol is followed. For more procedure details and typical results refer to Fenech and Neville [21] and Surrales et al. [32].

1.9. CBMN assay in other cell culture systems

1.9.1. Whole blood cultures for human lymphocytes

The CBMN assay in human lymphocytes can also be performed using whole blood cultures. Typically 0.4–0.5 ml of whole blood is added to 4.5 ml of culture medium (e.g., RPMI 1640) supplemented with fetal calf serum containing L-glutamine, antibiotics

(optional) and PHA. Cyt-B is added at 44-h post PHA stimulation. The recommended optimal concentration of Cyt-B for accumulating binucleated cells in whole blood cultures is 6 μg/ml [33]. The binucleated lymphocytes are harvested 28 h after adding Cyt-B, hypotonically treated with 0.075 M KCl to lyse red blood cells and fixed with methanol:acetic acid prior to transfer to slides and staining (it is also possible to smear the cells on the slides first and then fix them after air-drying). As an alternative it is also possible to isolate the binucleated lymphocytes directly from the whole blood culture using Ficoll gradients and then transfer cells to slides by cytocentrifugation prior to fixation and staining (unpublished observation) which precludes the requirement for hypotonic treatment and enables optimal preservation of the cytoplasm.

1.9.2. Murine lymphocyte cultures

Lymphocytes are isolated either from the spleen or peripheral blood and cultured according to the procedures described by Fenech et al. [34]. Because murine lymphocytes have shorter cell division cycles than human lymphocytes it is essential to add Cyt-B no later than 18 h after stimulation by mitogen and to harvest the cells 20 h later. Depending on the culture conditions, it is possible to obtain good binucleate ratios even at 72-h post mitogen stimulation.

1.9.3. Other primary cell cultures

The CBMN assay can be readily adapted to other primary cell types to assess DNA damage induced in vitro, in vivo or ex-vivo. The most important points to remember are (a) to ensure that MNi are scored in the first nuclear division following the genotoxic insult and (b) to perform preliminary experiments to determine the concentration of Cyt-B at which the maximum number of dividing cells will be blocked at the binucleate stage. It is also important to remember that Cyt-B may take up to 6 h before it starts to exert its cytokinesis-blocking action (unpublished observation). When using established or primary cell lines from dividing cell populations it is usual to add Cyt-B shortly after exposure to genotoxin to capture all cells undergoing their first nuclear division as binucleated cells — this usually requires an incubation period of about 24 to 48 h, depending on the cell cycle time, before harvesting the cells. Attached cells can be trypsinised and then prepared by cytocentrifugation as described for human lymphocytes. Specific methods have been described for use with nucleated bone-marrow cells [14], lung fibroblasts [35], skin keratinocytes [36] and primary tumour cell cultures [13]. It is generally more practical to assess in vivo induction of micronuclei by blocking cytokinesis in dividing cells after the cells have been isolated from the animal and placed in culture medium in the presence of Cyt-B; this approach has proven to be successful with a variety of cell types including fibroblasts, keratinocytes and nucleated bone-marrow cells.

1.10. Micronucleus assay in cell lines with or without cytokinesis-block

There is some debate that Cyt-B, used to accumulate binucleated cells, may interfere with the expression of MN [28]. Studies with normal cells do not show an induction of MNi by Cyt-B or a dose–response effect of Cyt-B with MN frequency in binucleated cells at doses that are usually used to block cells in cytokinesis [10,37–39]. A recent study suggests that MN expression induced by spindle poisons may be less than expected in the cytokinesis-blocked BN cells because of pole-to-pole distance shortening which may increase the probability of re-inclusion of lagging chromosome fragments or whole chromosomes back into a nucleus but this did not diminish the effectiveness of the CBMN assay [40].

There has been an increased interest in exploring further the possibility of performing the in vitro MN assay without Cyt-B to minimise the possible confounding effect of Cyt-B while running the potential risk of obtaining a false negative result because of inadequate control of cell division kinetics, i.e., inhibition of nuclear division inhibits micronucleus expression. While the evidence of obtaining a false positive result with the CBMN assay in normal cells is lacking, there is already adequate evidence that performing the MN assay in a manner that does not account for inhibition of nuclear division can lead to false negative results or an underestimate of MN induction in human lymphocyte cultures [10,11,41] and an example of this defect of MN assays without Cyt-B is shown in Fig. 9. Nevertheless, recent studies comparing the micronucleus assay with or without Cyt-B suggest that if cell lines with good growth characteristics are used and culture and nuclear

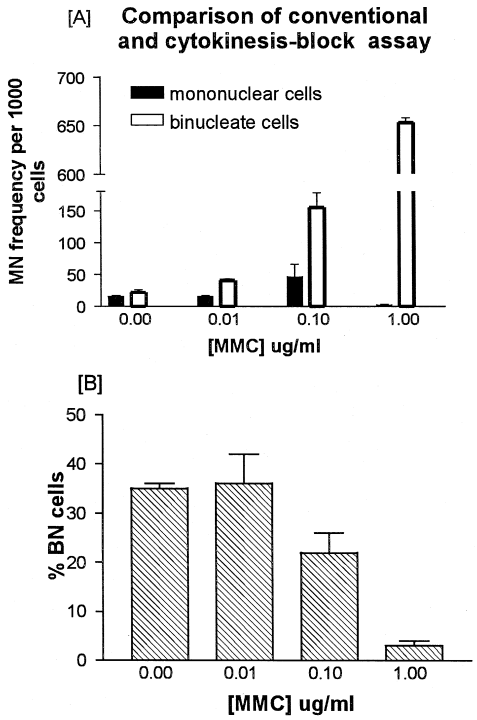

Fig. 9. [A] Comparison of the micronucleus dose–response in human lymphocytes exposed in vitro in $G_1/S/G_2$ to mitomycin-C (MMC) measured either in mononucleated cells in cultures without Cyt-B (solid black bars) or in binucleated cells in cultures with Cyt-B (white bars). [B] The level of dividing cells assessed by measuring the percentage of binucleated cells in the cytokinesis-blocked cultures. It is evident that the assay without Cyt-B underestimates the extent of genetic damage induced by MMC, particularly at doses that inhibit nuclear division. The data represent the mean $\pm$ 1 SE of three replicate cultures.

division conditions are optimal it is possible to obtain comparable results between the CBMN assay and the MN assay without Cyt-B when strong clastogens are tested [42,43]. A mathematical model of MN expression predicts (1) that scoring MN in BN cells is the most reliable way of determining micronucleus frequency and (2) scoring MN in mononucleated cells in cultures without cytokinesis-block is likely to generate false negative results when nuclear division is significantly inhibited by the chemical tested or the culture conditions do not allow an optimal number of dividing cells [44]. Consequently, results for micronucleus frequency obtained by scoring micronuclei in mononucleated cells in cultures without Cyt-B cannot be considered conclusive and that a negative result with this system should be confirmed using the CBMN assay.

2. Molecular techniques for measuring chromosome loss in micronuclei and non-disjunction

To take full advantage of the ability of the CBMN assay it is essential to distinguish between MNi originating from whole chromosomes or acentric fragments. This is best achieved by using probes that are specific for the centromeric DNA or antibodies that bind to the kinetochore proteins that are assembled at the centromeric regions of active chromosomes. The use of MN size as a discriminant is not recommended for human cells or other cell types in which the size of chromosomes is heterogenous because a small MN may contain either a fragment of a large chromosome or a whole small chromosome. The simplest and least expensive technique to use is the anti-kinetochore antibody method [45] but this approach does not distinguish between unique chromosomes and may not detect chromosome loss occurring due to absence of kinetochores on inactive centromeres [46]. The use of in situ hybridisation (ISH) to identify centromeric regions is more expensive and laborious but it can provide greater specificity; for example, centromeric probes for unique chromosomes can be used which also enables the detection of non-disjunctional events (i.e., unequal distribution of homologous chromosomes in daughter nuclei) in binucleated cells [17]. In this chapter, only the kinetochore antibody method will be described. For details on the use of centromere detection by ISH refer to the papers by Farooqi et al. [17], Hando et al. [18], Ehajouji et al. [23,47] and Schuler et al. [24]. The types of results that can be expected with the various techniques are illustrated in Fig. 10.

2.1. Kinetochore detection in MNi in the CBMN assay

2.1.1. Slide preparation

In this technique BN cells are accumulated as described in the standard CBMN assay, transferred to a slide using a cytocentrifuge, air-dried for 5 min and fixed in methanol for 10 min and air-dried again. At this stage slides may either be processed immediately

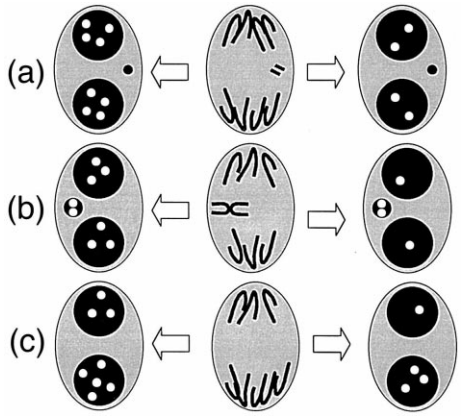

Fig. 10. The use of molecular techniques for identifying (a) a micronucleus originating from a lagging acentric chromosome fragment, (b) a micronucleus originating from a lagging whole chromosome and (c) non-disjunction of a chromosome leading to aneuploid daughter nuclei. The white spots in the nuclei and micronuclei of the binucleated cells on the left of each panel show the centromeric or kinetochore pattern of staining when pancentromeric probes or kinetochore antibodies are used. The white spots in the nuclei and micronuclei of the binucleated cells on the right of each panel show the pattern of centromeric staining when a centromeric probe specific to the chromosomes involved in micronucleus formation or non-disjunction events is used. The example shown is for a hypothetical cell with only two pairs of chromosomes.

or stored for a maximum of 3 months in a sealed desiccated box in a nitrogen atmosphere above liquid nitrogen. For detection of kinetochores the stored slides are removed from the nitrogen atmosphere and allowed to equilibrate at room temperature within the sealed box.

2.1.2. Kinetochore detection

The anti-kinetochore sera may either be obtained commercially or from an immunology clinic that has serum samples from scleroderma patients of the CREST subtype [48]. Use of the latter sera would require Human Ethics approval and consent from the donor patient. The sera should be tested on slides of metaphase spreads of cultured cells using a rabbit FITC-conjugated secondary anti-human IgG antibody and examined by fluorescence microscopy. Only sera that appear to react exclusively with kinetochores on metaphase chromosomes should be selected for the assay.

The use of FITC-conjugated secondary antibody to visualise kinetochores is a direct technique but requires the use of a fluorescence microscope and non-permanent slide preparations; the fluorescence technique has been described in detail elsewhere [45]. An alternative procedure is to use an immunoperoxidase staining method that allows permanent slide preparations to be obtained [49] which is more practical for routine screening and is described in the next paragraph.

In the immunoperoxidase technique, fixed slides are incubated overnight at 20°C in a humidity chamber with the primary anti-kinetochore antibody diluted 1/40 in Tris–saline buffer, pH 7.6 (6.0 g Tris–base/l saline). Negative control slides are exposed to the diluted serum of a normal healthy individual. The following day the slides are washed by dipping for 30 s in the same Tris–saline buffer used to dilute the antibody. Slides are then drained without drying, and incubated for 3 h with peroxidase-labelled rabbit anti-human IgG. Again, slides are then drained without drying in preparation for the peroxidase histochemical reaction.

The histochemical method that gives best contrast is the nickel chloride/imidazole modification of the standard diaminobenzidine (DAB) reaction which produces a black precipitate [50,51]. The reaction mixture consists of the following: 1 ml of DAB (1 mg/ml in Tris base buffer stock, 60.5 g/l, pH 7.6), 3 ml of Tris–base buffer stock pH 7.6 (60.5 g/l), 25 μl of $NiCl_2$ solution (8% solution in Tris–base buffer stock prepared immediately before use), 40 μl of 0.1 M imidazole and 10 μl of 30% hydrogen peroxide solution.

The reaction mixture is prepared just before use and applied immediately to slides through a 0.22-μm filter to minimise non-specific precipitation on the slides. Slides should be stained in batches including a slide with the negative control serum. The reaction is allowed to proceed for 1 min at 20°C and then stopped by draining the slides and rinsing in water. The slides are then air-dried, counterstained with the nuclear stain Neutral Red (0.1% in distilled water) for 30 s, washed in water, air-dried and mounted to give permanent preparations.

2.1.3. Scoring procedure

Scoring of kinetochore status of MNi is restricted to those binucleated cells in which a minimum of 20 kinetochores within each nucleus is observed. A minimum of 100 MNi should be classified according to whether they contain kinetochores or not and the

number of kinetochores within each MN should be noted. The final value for the proportion of MNi with kinetochores is determined by the following formula:

$$[P_s - P_c]/[1 - P_c],$$

where P_c is the proportion of MNi that has a positive peroxidase reaction in slides exposed to normal control serum and P_s is the proportion of MNi that have a positive peroxidase reaction in slides exposed to anti-kinetochore serum.

3. Treatment schedules for in vitro genotoxicity testing

Ideally each chemical should be tested for its genotoxic potential at the various stages of the cell cycle. Because human peripheral blood lymphocytes are in the G_0 phase when collected they are ideal for assessing damage at this stage. However, cells are expected to be more sensitive to genotoxic effects during S phase, G_2 phase and M phase and for this purpose it essential to expose cell cultures when most cells are dividing. Because MN expression requires one nuclear division to be completed the period between treatment and harvest time has to allow for this.

With human peripheral blood lymphocytes treated in G_0 it is necessary to accumulate binucleated cells as early as possible and for as long as possible to ensure that even cells experiencing mitotic delay are examined. Typically, the standard protocol of adding Cyt-B at 44 h and harvesting cells at 72 h should suffice for this purpose. However, it is equally practical to add Cyt-B at 24 h and harvest cells at 96 h which may maximise the number of late dividing cells available for analysis.

If treatment of cells in S, G_2 and M phases is required then exposure to the chemical should occur during logarithmic growth phase of the culture, followed shortly afterwards with Cyt-B to accumulate dividing cells, and cells are then harvested between 6 h and 24 h later depending on the stage of the cell cycle that is being examined. At the very early harvest times mainly cells exposed in G_2 or late S phase are accumulated as binucleated cells whilst at the later harvest time cells exposed in all stages of the cell cycle are blocked in the binucleate stage. Thus, the harvest time relative to Cyt-B addition would affect the type of cell examined.

Typical schedules for use of the CBMN assay for in vitro genotoxicity testing are summarised in Table 2. The use of a metabolic activation system such as S9 mix should be considered as an option when testing

Table 2
Typical protocols used for testing micronucleus inducion by a chemical or radiation*

Culture time (h)	Peripheral blood human lymphocytes				Cell lines in log phase
	CBMN assay G_0 exposure	CBMN assay G_1/S exposure	CBMN assay G_1/S/G_2/M exposure	CBMN/ARA assay, G_0 exposure	CBMN assay G_1/S/G_2/M exposure
−4	add test agent			add test agent	add test agent
0	add PHA	add PHA	add PHA	(i) add PHA, (ii) add ARA	add Cyt-B
16				(i) wash out ARA, (ii) fresh medium with IL-2 and DC	
20		add test agent			
24					harvest cells
44	add Cyt-B[a]	add Cyt-B[a]	add test agent	add Cyt-B[a]	harvest cells
48			add Cyt-B		
72	harvest cells	harvest cells	harvest cells	harvest cells	
96	harvest cells	harvest cells	harvest cells	harvest cells	

*The proposed protocols assume that the test agent is retained in the culture medium even after Cyt-B is added. However, it may also be desirable to remove test chemical by replacing culture medium (a) after a brief exposure period to test chemical or (b) just prior to addition of Cyt-B. In the latter case IL-2 should be added to fresh medium for lymphocyte cultures. ARA=cytosine arabinoside; Cyt-B=cytochalasin-B; DC=deoxycytidine; IL-2=interleukin-2; PHA=phytohaemagglutinin.

[a]Alternatively Cyt-B could be added at 24 h.

new chemicals but this could limit the exposure period due to the possible cytotoxicity of S9 to the target cells. A better option may be the use of metabolically competent cells such as genetically modified MCL-5 cells [52].

4. Future developments

It is evident that the in vitro micronucleus assay has evolved into a robust assay for genetic damage with applications in ecotoxicology [53], nutrition [54], radiation sensitivity testing both for cancer risk assessment [55] and optimisation of radiotherapy [13,56], biomonitoring of human populations [57] and importantly testing of new pharmaceuticals and agrichemicals [27,28]. There is little doubt that there is a need for an automated scoring system for quicker and more reliable data acquisition which would ideally be based on the scoring of slides also prepared for visual scoring — this should enable consistent results to be obtained that are not influenced by the inter-individual and temporal variability of human scorers. For this goal to be achieved it is essential that scoring criteria are well developed and that a robust slide preparation protocol be put in place and that slide preparations be permanent so that they can be re-examined visually if necessary. Currently image analysis systems have been developed for automated scoring of micronuclei in mammalian cells [58–62] but these systems do not take account of other important events such as necrosis, apoptosis and cytostasis which are essential for the correct interpretation of the result obtained [26]. In the future we should expect to have an automated system that can score reliably the various end-points possible with the cytokinesis-block micronucleus assay outlined in this paper.

Finally it is also essential to keep abreast of more recent developments in our understanding of micronucleus formation and events that may alter expression of this end-point. Some notable examples are (a) the formation of micronuclei as a result of gene amplification in which the cell eliminates excess amplified DNA directly from the nucleus, during S phase, into a micronucleus produced by nuclear budding [63]; (b) the observation that treatment with specific mitotic spindle inhibitors may cause mitotic slippage leading to polyploid nuclei and micronuclei and therefore implicating that it may be useful to score not only MNi in binucleated cells but also MNi in mononucleated cells in cytokinesis-blocked cultures [47] and (c) the possible elimination of micronucleated cells and micronuclei by apoptosis [64,65].

All of the above points to the fact that the full potential of the in vitro cytokinesis-block micronucleus assay is readily achievable once all the morphological end-points of cytotoxicity, cytostasis and DNA damage are integrated into the system.

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