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HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures☆

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Abstract

Criteria for scoring micronuclei and nucleoplasmic bridges in binucleated cells in the cytokinesis-block micronucleus assay for isolated human lymphocyte cultures are described in detail. Morphological characteristics of mononucleated cells, binucleated cells, and multinucleated cells as well as necrotic and apoptotic cells and nuclear buds are also described. These criteria are illustrated by a series of schematic diagrams as well as a comprehensive set of colour photographs that are of practical assistance during the scoring of slides. These scoring criteria, diagrams and photographs have been used in a HUman MicronNucleus (HUMN) project inter-laboratory slide-scoring exercise to evaluate the extent of variability that can be attributable to individual scorers and individual laboratories when measuring the frequency of micronuclei and nucleoplasmic bridges in binucleated cells as well as the nuclear division index. The results of the latter study are described in an accompanying paper. It is expected that these scoring criteria will assist in the development of a procedure for calibrating scorers and laboratories so that results from different laboratories for the cytokinesis-block micronucleus assay may be more comparable in the future.

1. Introduction

The HUman MicronNucleus (HUMN) project is an international collaborative project currently involving more than 30 laboratories world-wide and is aimed at improving the application and understanding of the lymphocyte cytokinesis-block micronucleus (CBMN) assay as an ex vivo measure of chromosome breakage and loss in individuals and human populations [1]. One of the key objectives of the HUMN project is to identify important methodological variables in the performance of the assay and the scoring of micronuclei

Abbreviations: BN, binucleated; CBMN assay, cytokinesis-block micronucleus assay; MN, micronucleus or micronuclei; MONO, mononucleated; MULT, multinucleated; NDI, nuclear division index; NPB, nucleoplasmic bridge or nucleoplasmic bridges; cyt-B, cytochalasin

☆ This paper has been adapted in part from Fenech [Mutat. Res. 455 (2000) 81–95].

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(MN) so that their confounding effects can be minimised. This will enable a better precision of the assay for detecting genotoxic events and therefore increase the reliability of the method for comparing DNA damage rates among individuals and populations and identify exposure conditions that induce increases in MN.

The CBMN assay is currently the most widely used method for measuring micronucleus frequency in human lymphocytes. The methods for performing the CBMN assay have been described recently [2]. In the first study completed by the HUMN project, databases of base-line MN frequencies in lymphocytes of 6583 subjects from 25 laboratories from 16 countries were compiled, compared and analysed to identify important, demographic, exposure and methodological variables that could influence the background MN frequency measured using the CBMN assay [3]. The results of this molecular epidemiological study showed that methodological variables and scoring criteria, accounted for a large proportion (75%) of the variation observed in the baseline MN frequency data. It therefore became apparent that a greater understanding of these methodological variables was needed and that scoring criteria (which accounted for 47% of the observed variation), in particular, needed to be standardised, clearly presented and explained.

Therefore, it was decided by the HUMN project coordinating group (http://HUMN.org) that an inter-laboratory slide-scoring exercise was needed to determine the extent of variability in MN frequency due to visual scoring of slides and staining method. The specific objectives, design and results of this study are described in detail in the accompanying paper [4]. Briefly, participating laboratories were provided with slides of cytokinesis-blocked isolated human lymphocyte cultures that were not hypotonically treated to score MN and nucleoplasmic bridge (NPB) frequency in binucleated (BN) cells and to determine the nuclear division index (NDI) based on the ratio of mononucleated (MONO), BN and multinucleated (MULT) cells. Most of the laboratories had no experience in scoring NPB, a biomarker of chromosome rearrangement [2]. To enable this study to be accomplished successfully it was necessary to develop a detailed set of scoring criteria together with a large set of schematic diagrams and photomicrographs of cells typically scored in the CBMN assay. These detailed instructions for scoring cells in the CBMN assay are described below and are based on a previously published procedure [2].

2. Scoring criteria

It is important to note that these scoring criteria and photomicrographs are applicable to isolated lymphocyte cultures that have not been treated by hypotonic solution. The photomicrographs in Figs. 1–9 were for lymphocytes stained with Diff-Quik (LabAids, Australia).

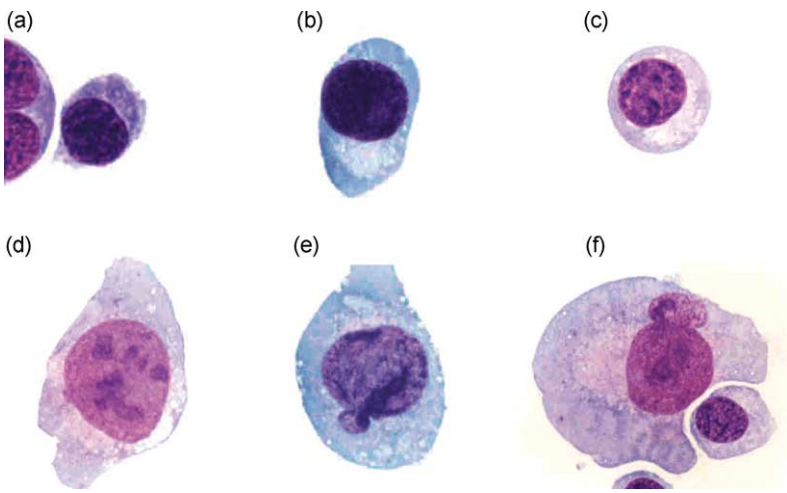

Fig. 1. (a–f) Photomicrographs of typical mononucleated (MONO) cells. Varying sizes of lymphocytes are shown with increasing size and increasing cytoplasmic volume/nuclear volume ratios. (e and f) Exhibit nuclear extrusions.

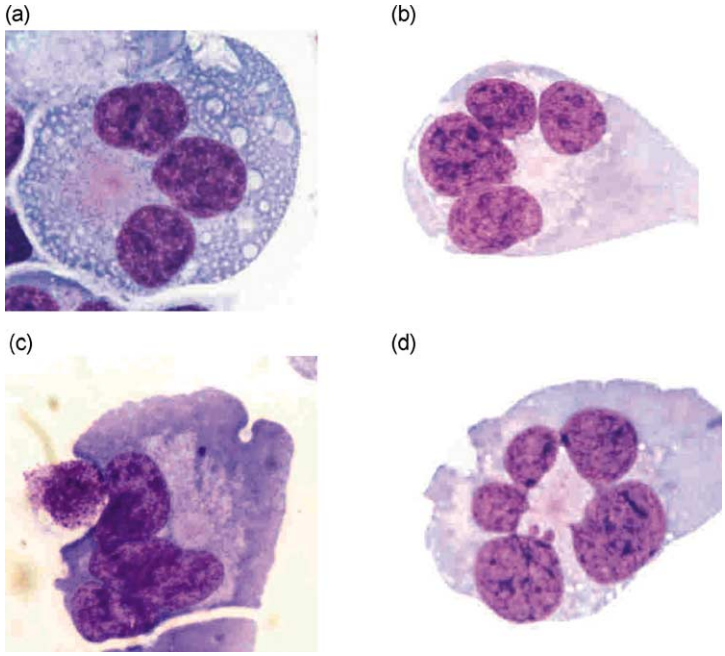

Fig. 2. (a–d) Photomicrographs of typical multinucleated (MULT) cells. The number of obvious nuclei may vary between three and eight nuclei depending on the number of nuclear divisions occurring since cyt-B addition. Sometimes it is difficult to determine the precise number of nuclei due to overlap and connections between nuclei.

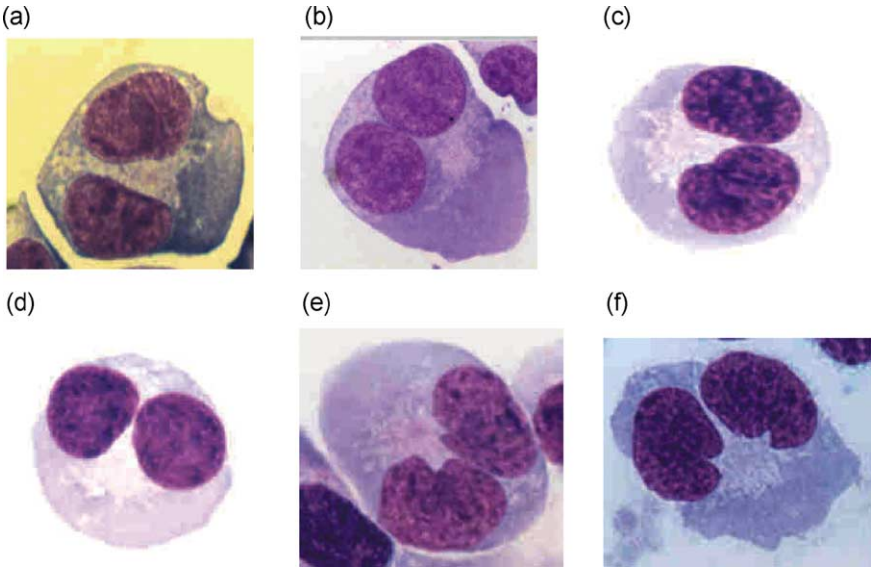

Fig. 3. (a–f) Photomicrographs of typical binucleated (BN) cells. (e and f) Illustrate cells in which the nuclei have an indentation; these are occasionally seen and should be considered as normal binucleated cells as they are not related to genotoxic exposure.

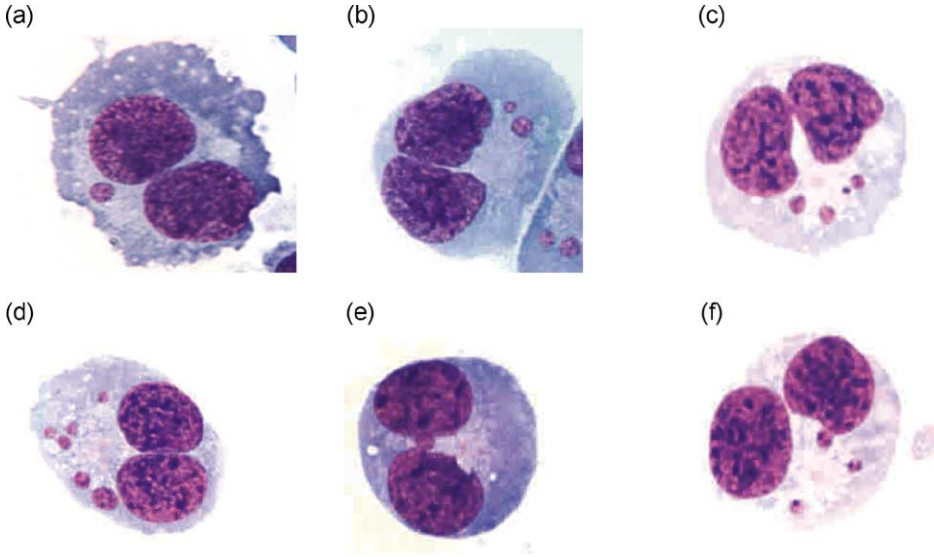

Fig. 4. (a–f) Photomicrographs of typical binucleated cells (BN) with micronuclei (MN). (e) Illustrates a micronucleus touching both nuclei.

2.1. Criteria for selecting binucleated cells which can be scored for the presence of micronuclei and nucleoplasmic bridges

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

1. The cells should be binucleated.
2. The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.

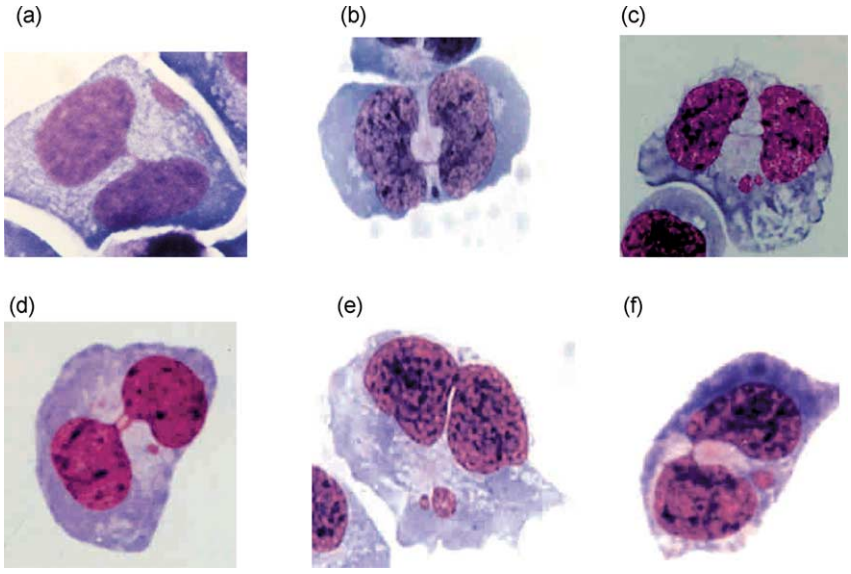

Fig. 5. (a–f) Photomicrographs of typical binucleated (BN) cells with both micronuclei (MN) and nucleoplasmic bridges (NPB). (c and d) Show a BN cell with two NPB. (e) Shows a BN cell with a very short NPB.

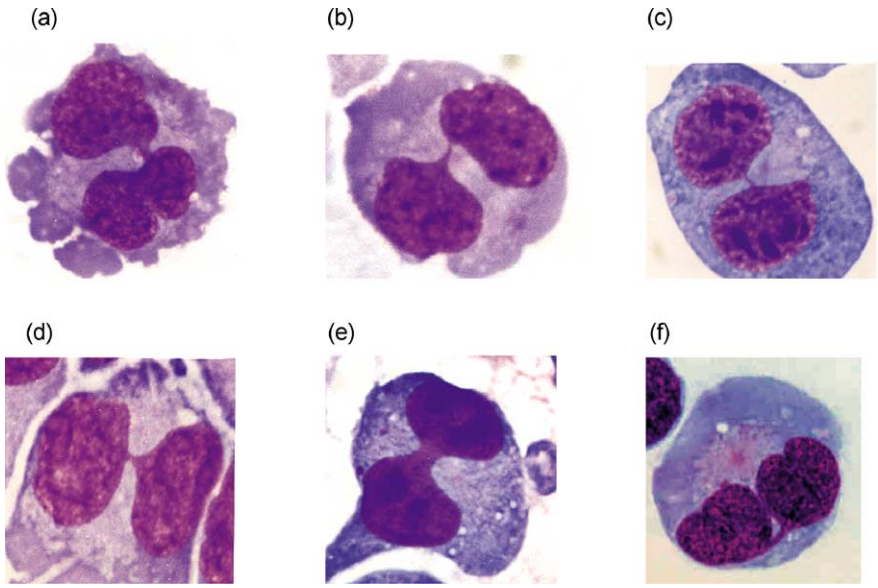

Fig. 6. (a–f) Photomicrographs of typical binucleated (BN) cells with nucleoplasmic bridges (NPB) but no micronuclei (MN). (e) Illustrates a relatively wide nucleoplasmic bridge.

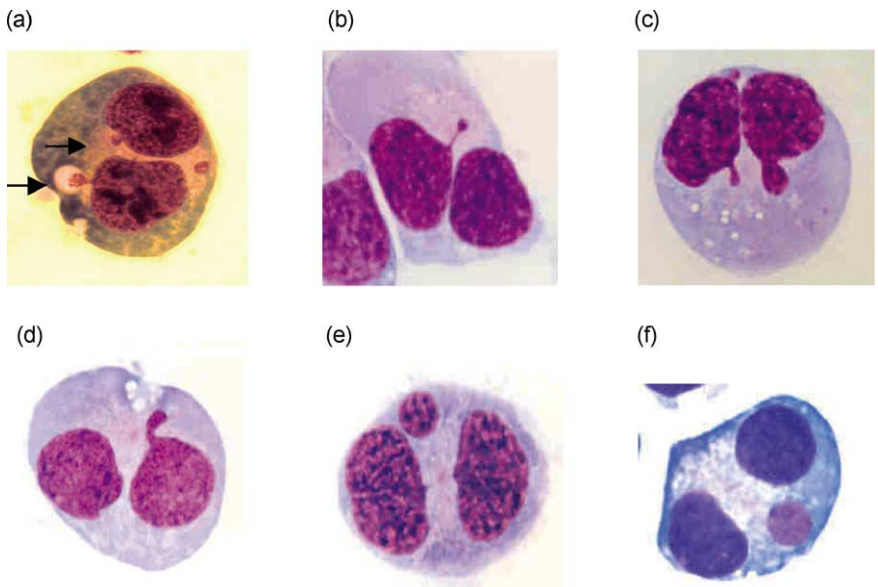

Fig. 7. (a–f) Photomicrographs of structures that resemble micronuclei (MN) but should not be classified as MN. (a) A BN cell with a micronucleus (on the right) and two nuclear buds (on the left) indicated by an arrow; the lower nuclear bud is surrounded by a vacuole. (b–d) BN cells with nuclear buds. (e and f) Trinucleated cells with one small nucleus which resembles a micronucleus but has a diameter greater than one-third the large diameter of the two main nuclei.

3. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.
4. The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter.
5. 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.
6. 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 be scored are illustrated in Figs. 3–6, 10 and 11. The cell types that should not be scored for micronucleus frequency include MONO and MULT (cells with more than two nuclei) cells, and cells that are necrotic or apoptotic (illustrated in Figs. 1, 2, 8, 9 and 12). It is important to note that nucleoplasmic bridges may be difficult to discern when nuclei in a binucleated cell are touching. Therefore, scoring of nucleoplasmic bridges may be best restricted to binucleated cells in which nuclei are clearly separated.

2.2. Criteria for scoring micronuclei (MN)

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

1. The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively.
2. MN are round or oval in shape.
3. MN are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles.
4. MN are not linked or connected to the main nuclei.
5. MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
6. MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

Examples of typical MN that meet the criteria set above are shown in Figs. 4 and 11. Examples of cellular structures that resemble MN but should not be classified as MN originating from chromosome breakage or loss are illustrated in Figs. 7 and 13. These structures include nuclear buds and small nuclei in trinuclear cells.

2.3. Criteria for scoring nucleoplasmic bridges (NPB)

NPB are sometimes observed in binucleated cells following exposure to clastogens and are thought to originate from rearranged chromsomes with more than one centromere, e.g. dicentric chromosomes. They have the following characteristics:

1. NPB are a continuous nucleoplasmic link between the nuclei in a binucleated cell.
2. The width of a nucleoplasmic bridge may vary considerably but usually does not exceed one-fourth of the diameter of the nuclei within the cell.
3. NPB should have the same staining characteristics of the main nuclei.
4. On rare occasions more than one nucleoplasmic bridge may be observed within one binucleated cell.
5. A binucleated cell with a nucleoplasmic bridge may or may not contain one or more micronuclei.

NPB are preferably scored in binucleated cells with clearly separated nuclei because it is usually difficult to observe an NPB when the nuclei are touching or overlapping. Binucleated cells with NPB often contain MN. Examples of binucleated cells with nucleoplasmic bridges are illustrated in Figs. 5, 6, 10C and D, and 11C.

2.4. Criteria for scoring apoptotic cells

1. Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries.
2. Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane.

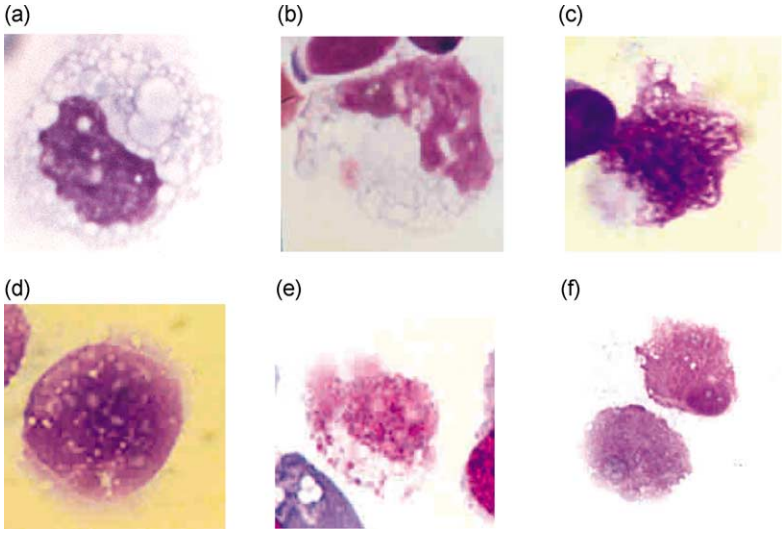

Fig. 8. (a–f) Photomicrographs of typical necrotic cells. (a–c) Cells in the early stages of necrosis when the cytoplasm is vacuolated and pale and the nucleus is only marginally intact. (d–f) Cells in the late stages of necrosis when the cytoplasm and cytoplasmic membrane are lost and the nucleus is clearly not intact and in a state of degeneration.

3. Staining intensity in the nucleus, nuclear fragments and cytoplasm is usually greater than in viable cells.

Figs. 9 and 12B illustrate typical examples of apoptotic cells.

2.5. Criteria for necrotic cells

1. Early necrotic cells can be identified by the presence of a pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus)

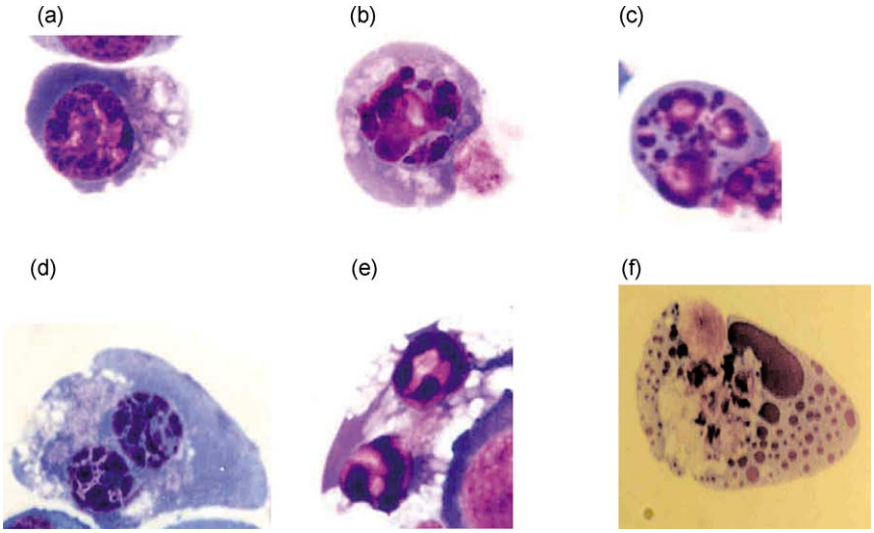

Fig. 9. (a–f) Photomicrographs of typical apoptotic cells. (a–c) Mononucleated cells at various stages of apoptosis showing condensed chromatin in the nucleus and a dark cytoplasmic staining. (d and e) A BN cell undergoing apoptosis. (f) A cell at the very late stage of apoptosis when the nuclear material has completely broken down within an intact cytoplasm and cytoplasmic membrane. An unusual feature about (d) is the presence of large vacuoles in the cytoplasm, suggesting a shift from apoptosis to necrosis.

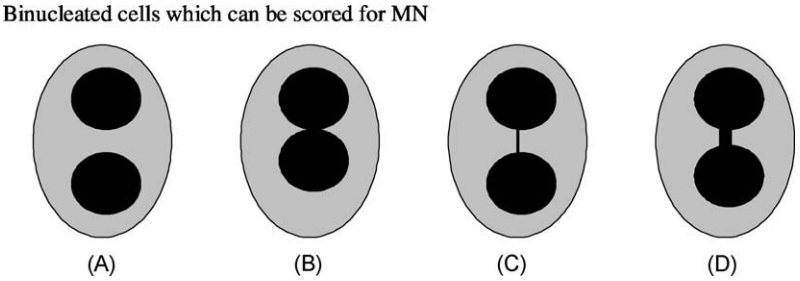

Fig. 10. Criteria for choosing binucleated cells in the cytokinesis-block micronucleus assay. (A) Ideal binucleated cell; (B) binucleated cell with touching nuclei; (C) binucleated cell with narrow nucleoplasmic bridge between nuclei; (D) binucleated cell with relatively wide nucleoplasmic bridge. Cells with two overlapping nuclei may be considered suitable to score as binucleated cells if the nuclear boundaries are distinguishable. Binucleated cells with touching or overlapping nuclei are usually not suitable for scoring nucleoplasmic bridges. Binucleated cells with more than one nucleoplasmic bridge are observed occasionally.

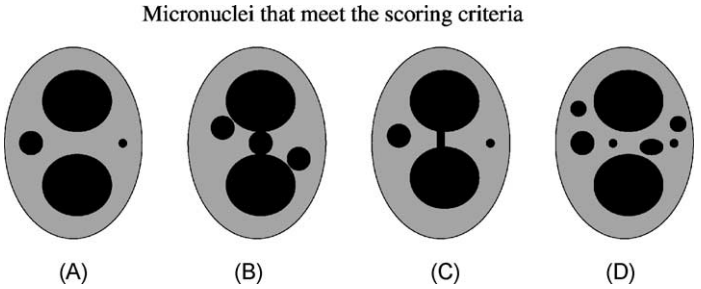

Fig. 11. Typical appearance and relative size of micronuclei in binucleated cells. (A) Cell with two micronuclei, one with one-third and the other one-ninth the diameter of one of the main nuclei within the cell. (B) Micronuclei touching but not overlapping the main nuclei. (C) A binucleated cell with 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.

and damaged cytoplasmic membrane with a fairly intact nucleus.
2. Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary.
3. Staining intensity of the nucleus and cytoplasm is usually less than that observed in viable cells.

Figs. 8 and 12C illustrate typical examples of necrotic cells.

3. Discussion

In the HUMN project inter-laboratory slide-scoring exercise, described in the accompanying paper [4], participants were only required to score MN and NPB frequency in BN cells, and NDI based on the ratio of MONO cells, BN cells and MULT cells, i.e. cells with more than two nuclei. However it has been shown that it is also practical to obtain a reliable score for the frequency of apoptotic and necrotic cells in isolated lymphocyte cultures without hypotonic treatment [5]. This adds important information to the assay because cells with damaged chromosomes may die rather than survive and express this damage as MN [5,6].

Another important development in the CBMN assay that is likely to alter the scoring procedure is the concept that MN should also be scored in MONO cells [6]. This is based on the fact that lymphocytes may already contain MN expressed as a result of in vivo cellular division. MN in MONO cells should indicate DNA damage that was present in the cells before they were put into culture with cytochalasin-B while BN cells may contain pre-existing MN as well as MN expressed during culture as a result of chromosome breaks

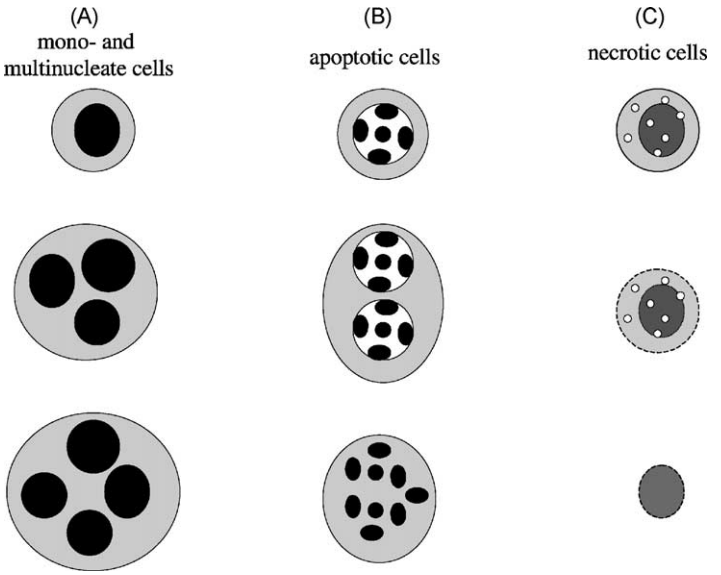

Fig. 12. The various types of cells that may be observed in the cytokinesis-block micronucleus assay, excluding binucleated cells. The cell types shown should not be scored for MN frequency: (A) viable mono-, tri- and quadrinuclear cells; (B) mono- (top) and binucleated (middle) cells at early stage of apoptosis when chromatin condensation has occurred but the nuclear membrane has not disintegrated and late stage apoptotic cells (bottom) with intact cytoplasm, and apoptotic chromatin bodies within the cytoplasm but no intact nucleus; (C) cells in early stages of necrosis (top, middle) showing vacuolisation, disintegration of cytoplasmic membrane and loss of cytoplasm with an intact nucleus and in late stages of necrosis (bottom) when cytoplasm is partially or completely lost, the nuclear membrane is visibly damaged and nuclear material is beginning to leak from the remnants of the nucleus.

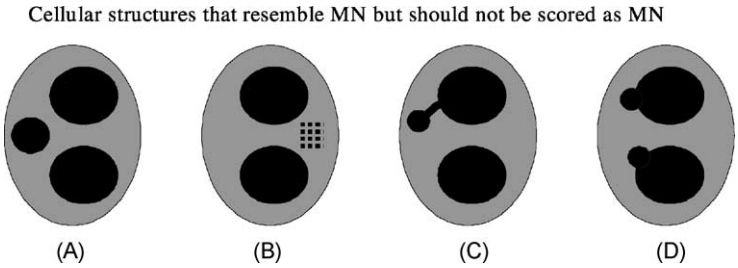

Fig. 13. Binucleated cells (or cells that resemble binucleated cells) may occasionally contain structures that resemble micronuclei but should not be scored as micronuclei originating from chromosome loss or chromosome breakage. These situations include: (A) a trinucleated cell in which one of the nuclei is relatively small but has a diameter greater than one-third the diameter of the other nuclei; (B) dense stippling in a specific region of the cytoplasm; (C) extruded nuclear material (nuclear buds) that appears like a micronucleus with a narrow nucleoplasmic connection to the main nucleus; (D) nuclear buds that have a wide and obvious nucleoplasmic connection with the main nucleus.

accumulated during G_0 phase in vivo. The scoring of MN in MONO cells in addition to scoring MN in BN cells should provide a more comprehensive procedure and ensure that all DNA damage events expressed as MN are recorded.

At the time of commencing the HUMN project in 1997 the significance of nuclear buds (Figs. 7a–d and 13C) and NPB in the CBMN assay was not clearly understood. However, evidence has been accumulating that nuclear buds arise from the elimination of amplified DNA [7–9] and possibly elimination of DNA-repair complexes [10]. NPB originate from dicentric chromosomes whose centromeres are pulled apart to opposite poles of the cell at anaphase [2,11].

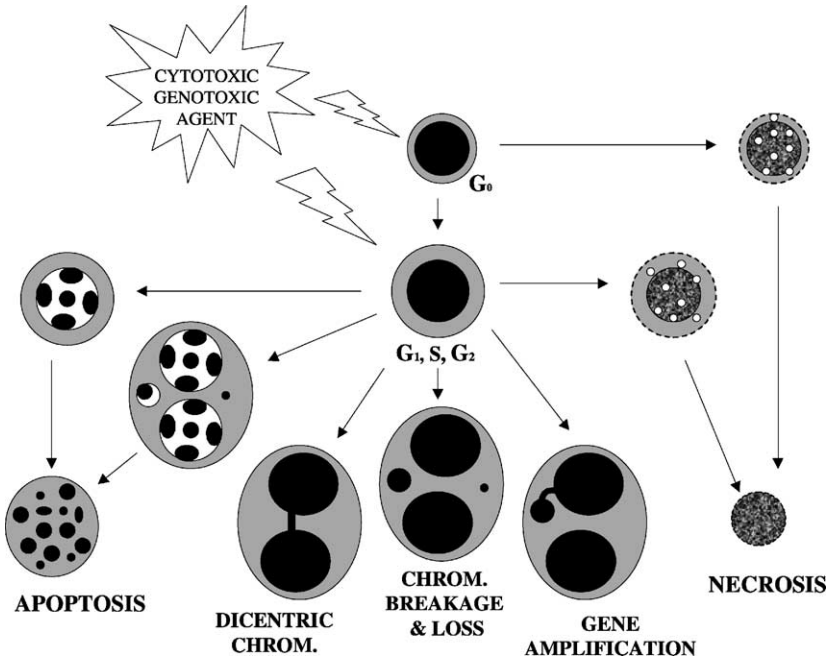

Fig. 14. The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents. Using these biomarkers within the CBMN assay it is possible to measure the frequency of chromosome breakage (MN), chromosome loss (MN), chromosome rearrangement e.g. dicentric chromosomes (NPB), gene amplification (nuclear buds), necrosis and apoptosis. In addition, cytostatic effects are readily estimated from the ratio of MONO, BN and MULT cells. Chromosome loss can be distinguished from chromosome breakage using pancentromeric probes or kinetochore antibodies [2]. In addition, non-disjunction (malsegregation of chromosomes) can also be measured in BN cells using chromosome-specific centromeric probes [2].

The inclusion of these chromosome damage markers in the CBMN assay, together with MN, necrotic and apoptotic cells permits the measurement of important complementary genotoxicity and cytotoxicity events, thereby the scope of the assay to measure chromosome breakage, chromosome loss, chromosome rearrangement, gene amplification, necrosis, apoptosis and cytostatic or cell proliferation effects. This comprehensive assessment of toxicity using the CBMN assay is illustrated in Fig. 14.

The criteria and photomicrographs described in this paper are not directly applicable to whole blood cytokinesis-block cultures because cells in that system are usually hypotonically treated to remove red blood cells. Hypotonic treatment is expected to eliminate necrotic cells which have a fragile cytoplasmic and nuclear membranes. Because hypotonic treatment also alters the observed morphology and structural detail of viable cells it will be necessary for the HUMN project to develop scoring criteria specifically for hypotonically treated lymphocytes. The increased use of the CBMN assay in combination with chromosome specific centromeric probes to measure chromosome loss and non-disjunction [2] also requires standardisation in terms of how probe signals are accepted, rejected and quantified if comparison of results from such tests among laboratories is to become feasible.

Finally, the results of the HUMN project slide-scoring study (accompanying paper [4]) have shown that even when laboratories score slides from the same cultures prepared in the same way, there is still a large difference between the results of different scorers and laboratories reflecting differences in visual discrimination of the structures scored in the assay and interpretation of scoring criteria. There is therefore clearly a need to calibrate scorers by means of either using (a) a set of standard reference slides or (b) digitised slide images that are planned for inclusion

on the HUMN project web site (http://HUMN.org). This will allow a scorer's or laboratory's data to be compared and "corrected" against an accepted international standard. Calibration of scorers is an important objective that is necessary before MN frequency data among populations and among laboratories can be reliably compared. These considerations also point towards the importance of the development of automated scoring procedures (e.g. flow cytometry, image analysis) which may eliminate some of the variability inherent in visual scoring.

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