Detection and characterization of micronuclei in a murine liver epithelial cell line, by application of the in vitro cytokinesis block MN assay and PRINS

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The cytokinesis block micronucleus assay was applied to murine cell line C6, derived from fetal liver, after an optimal protocol had been designed. Micronucleus frequencies were assayed after exposure to three concentrations of colcemid or diepoxybutane. Two-colour primed in situ DNA synthesis (PRINS) was applied to simultaneously label telomeric and centromeric (minor satellite DNA) sequences. Both chemicals induced a highly significant increase in MN and the effect was dose dependent. Diepoxybutane did not appear to significantly increase the frequency of centromere-positive micronuclei. Colcemid, as expected, induced high frequencies of centromere-positive micronuclei at all concentrations tested; in addition a significant increase in centromere-negative micronuclei was observed at 10^{-5} M. Many centromere-positive micronuclei carried three or four telomeres, thus indicating that a duplicated (non-disjoined) chromosome with two chromatids was contained in the micronucleus. This observation leads to the conclusion that micronuclei deriving from missegregation could be due to errors occurring before the onset of anaphase. The results obtained on C6 cells are in good agreement with those obtained on other cell systems, indicating that this cell line can be considered for in vitro aneuploidy evaluation.

Introduction

Micronuclei (MN) containacentric fragments or whole chromosomes that were excluded from daughter nuclei at mitosis. They are formed spontaneously or after exposure to genotoxic agents (Heddle et al., 1991). Cytochalasin B (Cyt B)-induced cytokinesis inhibition was proposed a decade ago to improve the the in vitro MN assay, since a sensible gain in information is obtained by identification of those interphase cells deriving from a single mitotic division (Fenech and Morley, 1985). Furthermore, after the introduction of immunofluorescence and molecular approaches for the localization of centromeric regions in MN, the power of the assay has increased further, leading to correct discrimination between clastogenic and aneugenic chemicals (Kirsch-Volders et al., 1997). The possibility of detecting both numerical and structural aberrations by means of a single in vitro assay is a unique feature of the MN test.

The development of in vitro mutagenesis assays which can ensure accurate results and are at the same time simple and fast is a critical step in reducing the use of laboratory animals. Two recent studies indicated that a high concordance of results (up to 80%) can be obtained between the in vitro MN test and the in vitro chromosome aberration assay (Miller et al., 1997, 1998); on the basis of these results the MN assay has been proposed as a replacement for the in vitro chromosome aberration assay (Miller et al., 1997, 1998). There is also a large consensus on inclusion of the MN assay in regulatory guidelines, coupled with molecular cytogenetics for aneuploidy detection (Albertini and Kirsch-Volders, 1997; Suralles and Natarajan, 1997). However, it has been pointed out that correct application of the MN methodology in testing strategies still requires a detailed standardization of protocols (Albertini and Kirsch-Volders, 1997; Suralles and Natarajan, 1997; Miller et al., 1998). There is concern about the possible disadvantages of treatment with Cyt B (Fenech, 1997; Suralles and Natarajan, 1997; Miller et al., 1998), since its side effects are still not fully understood. In fact, several authors have reported that Cyt B induces synergistic effects or interactions with aneugens (reviewed by Miller et al., 1998).

Primed in situ DNA synthesis (PRINS) is a new methodology for the cytogenetic localization of DNA sequences. After it was proposed a few years ago (Koch et al., 1989; Gosden et al., 1991) it has become progressively more popular and in different papers it was reported to give better results than FISH (Pellestor et al., 1996; Russo et al., 1996a,b). We first applied PRINS for MN characterization by tandem labelling with mouse minor and major DNA satellites (Russo et al., 1996a) and two-colour labelling of centromeric and telomeric sequences in mouse splenocytes (Russo et al., 1996b). Using PRINS, a very accurate labelling of telomeres in MN was obtained with respect to the performance of the FISH methodology (Miller and Nüsse, 1993; Schriever-Schwemmer and Adler, 1994). PRINS allowed, in particular, identification of three types of MN: those induced from a chromosome break event (expected to be C-, one telomere); those induced from missegregation of a whole chromatid (expected to be C+, two telomeres); those induced from missegregation of a whole chromosome without chromatid splitting (expected to be C+, four telomeres).

The C6 epithelial cell line was established from mouse fetal liver. These cells have a stable karyotype with a nearly diploid chromosome content and were shown to retain xenobiotic metabolizing activity (both oxidative and post-oxidative) from passage 40 to 90 (Paolini et al., 1991). For these reasons, C6 cells may represent a useful system for in vitro testing. C6 cells have already been used for MN evaluation without cytokinesis block (Canova et al., 1996). In this paper this cell line was used with the following purposes: (i) to describe the optimal cytokinesis block protocol for MN detection in the C6 cell line; (ii) to verify if aneugenic chemicals are correctly detected in this cell system, in particular to confirm on a different cell type, with different chemicals and at different concentrations, that C\(^+\) MN may harbour non-disjoined

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Berlin, Germany) supplemented with 7.5% fetal calf serum (FCS) (Biospa, Netherlands). Cells were set up, in order to expose exponentially growing cells. Cyt B was administered 20 h later. The compound, stored at 4°C, was diluted immediately before use in HBSS, in order to give

8–80 µl/ml culture medium. Since DEB is highly volatile, treated cells were cultured in tightly sealed flasks.

The definitive schedules of treatment are summarized in Figure 1.

After harvesting, cells were centrifuged at 1000 r.p.m. for 10 min and resuspended in a small volume of DMEM at a concentration of ~3×10^5 cells/ml. Cell preparations were obtained by cyto centrifuging 100 µl of the suspension per slide. For this step, thoroughly cleaned slides and a Shandon III cytocentrifuge were used; cells wereseeded at 800 r.p.m./min. Immediately after air drying, slides were fixed in absolute ethanol at −20°C and then stored under the same conditions until used.

5-Bromodeoxyuridine (BrdU) labelling

5-BrdU (Sigma) incorporation was used to evaluate the proportion of binucleated cells in S phase at harvesting. Incubation was carried out during the last 2 h of culture, at a final concentration of 10 µg/ml on cultures set up in parallel to those chemical tested plus one matched control culture were used. After harvesting, slides were prepared as described above; cell suspensions and slides were preserved as much as possible from light.

PRINS

To prepare interphase cells for the PRINS reaction, a proteolytic treatment was found to be necessary: slides were air dried, rehydrated in PBST (PBS, 0.1% Tween 20) for 15 min and incubated with pepsin (5 µg/ml in 0.01 N HCl, pH 3) (Roche Molecular Biochemicals, Mannheim, Germany) for 5 min at 37°C. After a brief washing step in milli-Q-purified water, slides were rinsed in 0.1 M Tris–HCl, pH 7.6, and finally dehydrated in ethanol (70, 90 and 100%) and air dried. Slides were then dipped in 0.1 M NaOH, 1 M NaCl on ice (90 s) to achieve cell denaturation, rinsed in 0.01 M Tris–HCl for 3 min and air dried. To verify the localization of centromeric and telomeric sequences in the C6 karyotype, PRINS was also carried out on chromosome preparations of C6 cells obtained by a conventional methodology. In this case a proteolytic treatment was not necessary and the denaturating step consisted of 60 min in 0.03 M NaOH, 1 M NaCl on ice. The two-colour PRINS reaction was carried out immediately after denaturation of preparations and it can be summarized as follows. In the first cycle the oligonucleotid 5′-TTAGGG3′ was used to label the mammalian telomere with digoxygenin-11-dUTP (Roche Molecular Biochemicals) as modified nucleotide. In the second cycle, an oligonucleotide corresponding to the mouse minor satellite sequence was used (Mitchell et al., 1993) and labelling was performed with biotin-16-dUTP (Roche Molecular Biochemicals). For each cycle, the reaction mixture consisted of ~300 pmol of the oligonucleotide of interest, 0.2 mM each dATP, dGTP and dCTP, 0.02 mM dTTP, 0.02 mM labelled deoxynucleotide, 5 U Taq DNA polymerase, 7.5 µM MgCl2 in a final volume of 50 µl PCR buffer. Nucleotides were obtained from Pharmacia (Sweden), the recombinant Taq DNA polymerase Dynazyme™ from Fynzymes Oy (Finland). Slides were briefly preheated (55°C) on the plate of a thermal cycler (“OmniGen equipment with an in situ block; Hybird, UK”). 12 µl of the reaction mixture were then placed on each slide and covered with an 18×18 coverslip and the edges were carefully sealed with nail polish. For the first PRINS cycle, the annealing and extension steps were, respectively, 53°C for 10 min and 72°C for 30 min. After cooling down the cycle, the coverslips were removed and the slides dipped in blocking solution (50 mM EDTA, 50 mM NaCl, pH 8) for 2 min at 72°C and then in 0.01 M Tris–HCl for 2–3 min. The second PRINS cycle was carried out under the following conditions: annealing at 50°C for 10 min, extension at 63°C for 30 min. After stopping the reaction in blocking solution, slides were washed twice (5 min each) in 4× SSC, pH 7.0, 0.1% Tween 20 and once in PBST, 0.5% non-fat dry milk. A mixture of fluorescein-conjugated anti-digoxygenin antibody (1:12 v/v; Roche Molecular Biochemicals) and Texas Red–Avidin (1:500 v/v; Vector Laboratories, Burlingame, CA) in PBST was used to detect the reaction signals (30 µl under a 20×20 coverslip, 30 min at 37°C in a moist chamber). After two washes in PBS, slides were mounted in an antifade solution (Vectorshield™, Vector Laboratories) containing 5 µg/ml DAPI (Roche Molecular Biochemicals) as counterstain.

Immunodetection of BrdU-labelled cells

Slides were removed from ethanol and air dried, denatured in 0.01 M NaOH, 1 M NaCl on ice (1 min) and then washed in 0.01 M Tris–HCl for 3 min and in PBST (5 min each). Preparations were then incubated with monoclonal anti-BrdU antibodies developed in mouse (Sigma), diluted 1:1000 in PBST (30 µl/slide, 30 min at 37°C). After two washes in PBST (5 min each), slides were incubated with fluoresceinated antibodies against mouse IgG (Roche Molecular Biochemicals) (1:100 v/v in PBST, 30 µl/slide, 30 min at 37°C), washed twice in PBS and mounted with DAPI/Vectashield® as reported above.

Microscopical and statistical analyses

For the preliminary experiments aimed to identify the optimal experimental design, Giemsa stained slides were analysed by light microscopy (using either

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**Materials and methods**

**Cell cultures**

C6 cells, growing as monolayers, were cultured in 25 cm² flasks at 37°C and 5% CO2 in Dulbecco’s minimum essential medium (DMEM) (Seromed, Berlin, Germany) supplemented with 0.8×10^6 cells/flask. In the above culture conditions the C6 cell line is unsplit) chromosomes, as originally found after mouse spleenocyte analysis (Russo et al., 1996b); (iii) to better understand the effects of Cyt B, especially when used together with chemicals acting on structures of the mitotic apparatus.

**Cytokinesis block: preliminary experiments**

To verify the optimal conditions for Cyt B (Sigma, Italy) exposure, treatments with two concentrations (3 and 6 µg/ml) and at three different time intervals (18, 20 and 24 h) were considered. Cyt B was administered 26 h after cultures were set up, in order to expose exponentially growing cells. Cyt B was dissolved in dimethyl sulfoxide (DMSO) (Sigma) and stored at −80°C as a stock solution of 2 µg/µl. Before administration, Cyt B was diluted to 0.5 µg/µl in phosphate-buffered saline (PBS).

In the same preliminary phase of the study, the two different colcemid concentrations (10−2 and 10−6 M) were assayed for co-treatment with Cyt B to verify possible concurrent effects of the drugs. Cells were exposed to Cyt B (3 µg/ml) and colcemid for 14 h, then washed and released from mitotic block in the presence of fresh medium containing Cyt B for the last 4 h of culture. A diagram summarizing the schedule of treatments in this pilot experiment is shown in Figure 1.

**MN assay**

Based on the indications obtained after preliminary experiments (previous paragraph), the following schedules of exposure were used for the two chemicals under investigation.

1. Colcemid (CAS no. 477-30-5; Sigma) was tested at 10−7, 10−6 and 10−5 M. The compound was dissolved in sterile distilled water at 100 µg/ml and stored at −20°C. Twenty-six hours after cultures were set up, 4–40 µl of colcemid solution were given per ml culture medium, according to the required final concentration. When necessary, the stock solution was further diluted immediately before treatment. After 5 h treatment, the monolayers were washed in Hank’s balanced salt solution (HBSS) and cultured for an additional 20 h in the presence of Cyt B (3 µg/ml) before harvesting.

2. Diepoxybutane (DEB) (CAS no. 298-18-0; Sigma) was tested at 10−7, 10−6 and 10−5 M. DEB and Cyt B were administered simultaneously, at 26 h after the beginning of culture; harvesting was 20 h later. The compound, stored at 4°C, was diluted immediately before use in HBSS, in order to give

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**Fig. 1.** Experimental design during a pilot experiment and the definitive schedule of treatments used in this study.
with colcemid at two different concentrations. Morphology of C6 cells after Cyt B treatment: cell preparations

Yield of mono-, bi-, tri- and tetranucleated cells obtained in C6 cultures after incubation with Cyt B for different time intervals and at different concentrations: (A) 3 µg/ml; (B) 6 µg/ml.

Fig. 2. Yield of mono-, bi-, tri- and tetranucleated cells obtained in C6 cultures after incubation with Cyt B for different time intervals and at different concentrations: (A) 3 µg/ml; (B) 6 µg/ml.

Fig. 3. Yield of mono-, bi-, tri- and tetranucleated cells obtained in C6 cultures after incubation with 3 µg/ml Cyt B and simultaneous treatment with colcemid at two different concentrations.

Fig. 4. Morphology of C6 cells after Cyt B treatment: cell preparations stained with Giemsa. (A) Microscopic field showing one binucleated and one trinucleated cell (a MN is visible in the trinucleated cell). (B) Microscopic field showing two adjacent binucleated cells, one of them carrying a micronucleus. The cell on the left shows overlapping main nuclei.

Table I. Results

The C6 cell line consists of one diploid and one triploid subpopulation, the latter corresponding to about 1/3 of the total. In the majority of C6 cells, all chromosomes were acrocentric, as in the original mouse karyotype; a small percentage of cells (<18%) carried a metacentric chromosome (Robertsonian translocation). PRINS analysis carried out to label centromeric and telomeric sequences on chromosome preparations did not reveal major rearrangements between chromosomes. One pair of chromosomes typically showed a duplication of the centromeric region, with a minor–major–minor satellite DNA array. The stability of the chromosome set in the C6 cell line was assessed immediately before their use for the MN assay and at the end of the experiments (at passages 58 and 65, respectively). The results are shown in Table I.

Pilot experiments were carried out to identify the optimal conditions for Cyt B treatment and exposure to genotoxic agents. The kinetics of binucleated cell production were evaluated after exposure of C6 cells to two Cyt B concentrations and at three different time intervals. For each exposure condition at least 1500 cells were scored, and the results are shown in Figure 2. Similar patterns were observed with the two concentrations (3 and 6 µg/ml) and the three time intervals tested (18, 20 and 24 h), which produced on average 20–30% mononucleated, 60–75% binucleated and 0–3% trinucleated cells (Figure 2a and b).

In parallel with the experiments described above, the efficiency of the cytokinesis block in the presence of a genotoxic agent was verified. For this purpose, exponentially growing C6 cells were treated with two different concentrations of colcemid (10−7 and 10−6 M) for 14 h, in the presence of 3 µg/ml Cyt B. Cells were then washed and cultured for an additional 4 h in fresh medium containing Cyt B only (in this pilot experiment the duration of Cyt B treatment was 18 h, as depicted in Figure 1). At least 1000 cells were scored per experimental condition, and the results are given in Figure 3. While in control cultures the observed percentages of mono-, bi- and trinucleated cells were in agreement with those found in the previous experiment (Figure 2A), an increase in mononucleated with respect to binucleated and the other cell types was found in response to colcemid treatment. This effect was
clearly dose dependent. In addition, cells with an irregular nuclear shape/size or nuclear multifragmentation were observed. To determine whether the increase in mononucleated cells in the population exposed to colcemid resulted from a mitotic block/delay induced by the chemical, the presence of MN was investigated in mono- and binucleated cells. The analysis showed that for each colcemid concentration, comparable MN frequencies were found in both cell types, with an increase of about 10 times the corresponding baseline (data not shown).

Based on these preliminary observations, the protocol for colcemid treatment was modified as described in Materials and methods. In addition, for all the following experiments the duration of Cyt B incubation was set at 20 h (Figure 1), to reduce to as low as possible the frequency of trinucleated cells (Figure 2). To minimize any side effect of Cyt B, 3 µg/ml were given throughout. Examples of bi- and trinucleated C6 cells are shown in Figure 4.

The frequencies of mono-, bi- and multinucleated cells and the frequency of MN in these cell types were recorded throughout to provide evidence of the accuracy of the cytokinesis block. Table II shows the percentage distribution of cells as a function of the number of nuclei. It should be noted that the relative percentage of mono-, bi-, tri- and tetrnucleated cells did not vary among the different treatment conditions with respect to control cultures. The proportion of cells showing nuclear fragmentation was significantly increased after exposure to colcemid (10^{-7} M, \( P < 0.005; 10^{-6} \text{ M, } P < 0.05; 10^{-5} \text{ M, } P < 0.01 \)), but not after treatment with DEB (Table II). In Table III, the observed frequencies of mono- and binucleated cells carrying MN (mean values and standard errors calculated from the replicate experiments) are indicated for each treatment condition. In control cultures, the average frequency of micronucleated cells was found to be 13.6 ± 4.2 per 1000 mononucleated cells and 11.3 ± 1.9 per 1000 binucleated cells. Statistical comparisons of means by Student’s t-test indicated that the frequency of micronucleated cells with MN was not affected by exposure of C6 cells to colcemid or DEB. However, a significant increase in binucleated cells with MN was observed after treatment with colcemid (10^{-7} M, \( P < 0.01; 10^{-6} \text{ M, } P < 0.05; 10^{-5} \text{ M, } P < 0.005 \)). This effect was dose related, as confirmed by linear regression analysis (\( P < 0.05 \)). As for treatment with DEB, simple comparison of means indicated a significant increase in MN only at the highest dose tested (10^{-5} M, \( P < 0.005 \)). However, linear regression analysis was also significant at \( P < 0.05 \), indicating the existence of a dose-related response of C6 cells to DEB exposure.

In Figure 5 and Table IV, the results from MN analysis in binucleated cells are further subdivided according to the presence of a centromeric region in the MN. MN were classified as C+ or C− only when adequate labelling of centromeric sequences was observed in the main nuclei. Otherwise, MN

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**Table I.** Chromosome analysis of C6 cell population before (passage 58) and after (passage 65) the end of the experiments here described

<table>
<thead>
<tr>
<th>Passage</th>
<th>No. metaphases scored</th>
<th>2n metaphases (range 35–50)</th>
<th>3n metaphases (range 51–74)</th>
<th>&gt;3n metaphases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total no. (%)</td>
<td>Modal no. (%)</td>
<td>With dicentric</td>
</tr>
<tr>
<td>58</td>
<td>108</td>
<td>67 (62.0%)</td>
<td>8a</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>102</td>
<td>65 (63.7%)</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

*One cell carried two dicentrics.

The number of dicentric chromosomes (the only structural rearrangement observed) is reported.

**Table II.** Relative percentages of mono-, bi- and multinucleated cells observed in C6 cultures as a function of the treatment

<table>
<thead>
<tr>
<th>Cells analysed</th>
<th>Mononucleated cells (%) ± SE</th>
<th>Binucleated cells (%) ± SE</th>
<th>Trinucleated cells (%) ± SE</th>
<th>Tetrnucleated cells (%) ± SE</th>
<th>Cells with fragmented nuclei (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>7138</td>
<td>26.12 ± 3.35</td>
<td>72.70 ± 3.20</td>
<td>0.47 ± 0.14</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>Colcemid, 10^{-7} M</td>
<td>3092</td>
<td>22.74 ± 1.21</td>
<td>74.76 ± 1.17</td>
<td>0.71 ± 0.03</td>
<td>0.05 ± 0.05</td>
</tr>
<tr>
<td>Colcemid, 10^{-6} M</td>
<td>5567</td>
<td>26.94 ± 0.37</td>
<td>69.49 ± 0.90</td>
<td>0.74 ± 0.24</td>
<td>0.07 ± 0.04</td>
</tr>
<tr>
<td>Colcemid, 10^{-5} M</td>
<td>4734</td>
<td>27.52 ± 1.60</td>
<td>66.15 ± 0.84</td>
<td>1.26 ± 0.13</td>
<td>0.10 ± 0.10</td>
</tr>
<tr>
<td>DEB, 10^{-7} M</td>
<td>4337</td>
<td>24.62 ± 3.71</td>
<td>73.66 ± 3.25</td>
<td>0.44 ± 0.01</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>DEB, 10^{-6} M</td>
<td>4282</td>
<td>21.64 ± 0.55</td>
<td>76.99 ± 0.33</td>
<td>0.34 ± 0.03</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>DEB, 10^{-5} M</td>
<td>5555</td>
<td>26.65 ± 2.87</td>
<td>72.14 ± 2.77</td>
<td>0.29 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
</tbody>
</table>

\(a P < 0.005 \) (Student’s t-test).
\(b P < 0.05 \) (Student’s t-test).
\(c P < 0.01 \) (Student’s t-test).

**Table III.** Frequencies of micronuclei observed in mononucleated and binucleated C6 cells after treatment with colcemid or DEB (averages and standard errors were calculated from the replicated experiments)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total With MN (% ± SE)</th>
<th>Binucleated cells Total With MN (% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1834 26 (13.6 ± 4.2)</td>
<td>5219 (11.3 ± 1.9)</td>
</tr>
<tr>
<td>Colcemid, 10^{-7} M</td>
<td>712 14 (18.8 ± 3.0)</td>
<td>2303 (28.7 ± 1.9)</td>
</tr>
<tr>
<td>Colcemid, 10^{-6} M</td>
<td>1500 17 (12.0 ± 3.8)</td>
<td>3868 (38.1 ± 5.7)</td>
</tr>
<tr>
<td>Colcemid, 10^{-5} M</td>
<td>1296 20 (15.3 ± 2.6)</td>
<td>3135 (48.1 ± 0.5)</td>
</tr>
<tr>
<td>DEB, 10^{-7} M</td>
<td>1089 18 (18.5 ± 7.0)</td>
<td>3176 (18.0 ± 4.1)</td>
</tr>
<tr>
<td>DEB, 10^{-6} M</td>
<td>933 11 (11.8 ± 0.2)</td>
<td>3293 (27.4 ± 9.6)</td>
</tr>
<tr>
<td>DEB, 10^{-5} M</td>
<td>1486 18 (12.4 ± 1.8)</td>
<td>4000 (41.5 ± 2.0)</td>
</tr>
</tbody>
</table>

\(a P < 0.01 \) (Student’s t-test).
\(b P < 0.05 \) (Student’s t-test).
\(c P < 0.005 \) (Student’s t-test).
Detection and characterization of micronuclei were regarded as ‘unclassifiable’. In Figure 5, the observed percentage of each category of MN with respect to their total number is shown for each treatment condition. In control cultures, C+ MN represented ~25% of the total, while for ~20% of MN a classification was not possible, because of inadequate quality of the fluorescent signals in the main nuclei. As can be seen from Figure 5, the main effect induced by colcemid in C6 cells was, as expected, an increased proportion of C+/H11001 MN (up to 57% of the total number of observed MN). In contrast, DEB induced an increase in the relative proportion of C− MN. For both chemicals tested, the proportion of unclassifiable MN fluctuated from 16 to 27%, a range also including the observed control value. Since unclassifiable MN represent a constant proportion whatever the treatment considered, they can be reasonably interpreted as a consequence of technical limitations of the labelling protocol. To better understand the effects induced by the two compounds under study, the C+/H11001 and C− MN frequencies were calculated per 1000 binucleated cells and each frequency was compared with the corresponding control value using Student’s t-test. Unclassifiable MN were not analysed in this way, as they do not represent a biological effect of treatment.

Concerning 353 colcemid, it can be noticed that besides its main aneugenic effect, this compound also induced a statistical increase in C− MN at the highest concentration tested (10−5 M, P < 0.005; Table IV). Analysis of data from the DEB experiments showed that the increase in MN observed at the highest dose tested was exclusively due to the contribution of C− MN (P < 0.005), while no increase in C+ MN was observed at any DEB concentration (Table IV).

When MN were classified according to the number of telomeric signals (Figure 6), a clear pattern was observed: the majority of C+ MN carried more than two signals, a minor proportion showed two telomeres only and only few of them carried one telomere or no telomeric signal at all (Figure 6, Fig. 6. Distribution of MN observed in binucleated C6 cells as a function of the number of visible telomeres (black columns) and of the presence of the centromeric region (white columns). tMN, total MN; uMN, unclassifiable MN.

![Fig. 5.](image-url) Relative proportion of C+ and C− MN and of MN observed in cells with an inadequate labelling pattern (unclassifiable MN), as observed under different treatment conditions in binucleated C6 cells.)

![Fig. 6.](image-url) Distribution of MN observed in binucleated C6 cells as a function of the number of visible telomeres (black columns) and of the presence of the centromeric region (white columns). tMN, total MN; uMN, unclassifiable MN.

**Table IV.** Frequencies of centromere-positive (C+) and centromere-negative (C−) MN in binucleated C6 cells after treatment with colcemid or DEB  

<table>
<thead>
<tr>
<th></th>
<th>No. cells analysed</th>
<th>No. MN (% ± SE)</th>
<th>C− MN (% ± SE)</th>
<th>C+ MN (% ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>5219</td>
<td>67 (12.3 ± 2.4)</td>
<td>36 (6.8 ± 0.6)</td>
<td>17 (3.0 ± 1.1)</td>
</tr>
<tr>
<td>Colcemid, 10−7 M</td>
<td>2303</td>
<td>74 (31.8 ± 1.6)</td>
<td>33 (13.6 ± 3.5)</td>
<td>22 (9.6 ± 0.4)</td>
</tr>
<tr>
<td>Colcemid, 10−6 M</td>
<td>3868</td>
<td>151 (42.8 ± 7.3)</td>
<td>39 (11.9 ± 3.4)</td>
<td>86 (23.6 ± 2.7)</td>
</tr>
<tr>
<td>Colcemid, 10−5 M</td>
<td>3135</td>
<td>183 (58.3 ± 0.7)</td>
<td>78 (25.0 ± 1.3)</td>
<td>74 (23.3 ± 3.3)</td>
</tr>
<tr>
<td>DEB, 10−7 M</td>
<td>3176</td>
<td>60 (19.3 ± 4.2)</td>
<td>32 (10.4 ± 4.1)</td>
<td>12 (3.8 ± 0.3)</td>
</tr>
<tr>
<td>DEB, 10−5 M</td>
<td>3293</td>
<td>93 (31.2 ± 11.5)</td>
<td>64 (21.5 ± 8.0)</td>
<td>14 (4.6 ± 1.2)</td>
</tr>
<tr>
<td>DEB, 10−3 M</td>
<td>4000</td>
<td>182 (45.5 ± 0.5)</td>
<td>123 (30.8 ± 2.3)</td>
<td>20 (5.0 ± 2.5)</td>
</tr>
</tbody>
</table>

The total number of MN may be different from the number of binucleated cells with MN, shown in Table III, because some cells carried more than one MN.  

*P < 0.01 (Student’s t-test).  

*P < 0.05 (Student’s t-test).  

*P < 0.005 (Student’s t-test).
see in particular panel A). However, C– MN did not show more than two telomeres, apart from a few exceptions (Figure 6, see in particular panel B). Rarely, MN with five telomeric signals were observed. An example of two-colour labelling of centromeric and telomeric regions in C6 binucleated cells is given in Figure 7.

5-BrdU labelling experiments gave the results summarized in Table V. In mononucleated cells, the percentage of labelled cells ranged from 11.4 to 20.4% in control cultures, the highest value corresponding to the prolonged time schedule used for testing colcemid effects (an additional 5 h of cell growth in the presence of the chemical, before Cyt B exposure). At harvesting, both chemicals were found to have produced a statistically significant increase in 5-BrdU-labelled cells, with respect to the observed control ($P < 0.005$; Table V). Notably, all observed MN in the mononucleated cell population were unlabelled, irrespective of whether they were associated with a labelled or an unlabelled main nucleus. Among binucleated cells, in untreated cultures only 2–3% of them showed at least one labelled nucleus at harvesting. After exposure to colcemid this proportion increased to ~14% ($P < 0.005$); treatment with DEB did not cause an increase in 5-BrdU-labelled binucleated cells. As for mononucleated cells, all observed MN were unlabelled.

Discussion

C6 cells were assayed for their possible use in an in vitro MN test. In the C6 cell line two subpopulations were found, the first with a diploid chromosome set, the second with a 3n chromosome number. The two subpopulations appeared quite stable with respect to their relative proportion and chromosome number. Furthermore, no structural chromosome aberrations were observed, apart from the presence of a metacentric chromosome in a minority of cells. The stability of the C6 karyotype, observed in this study up to passage 65, further confirms previous information reported by Paolini et al. (1991).

Concerning the use of Cyt B and its possible side effects, in this study it was observed that simultaneous administration of Cyt B and colcemid caused a pronounced interference of their activities. The yield of binucleated cells dropped to 40% when cells were exposed to $10^{-7}$ M colcemid and reached only 30% at a concentration of $10^{-6}$ M. In contrast, in control cultures 70% of binucleated cells were found. A simple explanation for the decrease in binucleated cells would be a possible mitotic block or delay induced by colcemid. However, this hypothesis must be rejected, since highly comparable MN frequencies were found in both mono- and binucleated cells. As a consequence of a successful protocol for cytokinesis arrest, mononucleated cells should represent those cells that did not undergo mitosis after exposure; accordingly, the MN frequency in this population is not expected to vary after treatment. To explain the increase in MN in mononucleated cells, it must be postulated that many of the dividing C6 cells can escape the cytokinesis block when Cyt B and colcemid are administered simultaneously. At the same time, MN can be formed as a consequence of colcemid exposure, leading to an increase in the MN frequency in the mononucleated subpopulation. With the alternative protocol under which the treatments with colcemid and Cyt B were not associated, the yield of binucleated cells was comparable with that obtained in control and DEB-treated cultures. In addition, after treatment with both chemicals the frequency of MN observed in monono-
colcemid and colchicine have already been reported. Proportions of C− MN in the C6 cell line as a response to treatment with
DEB have been previously described for correct evaluation of the main mechanism of action of chemicals.

The effects of DEB exposure have been previously described by different studies in vivo and in vitro and the hypothesis that DEB may have aneugenic potential has been raised (Xiao et al., 1996a,b; Xi et al., 1997; Murg et al., 1999). In this study an increase in C+ MN was not observed at the doses tested. It should be noted, however, that the variability between replicates was rather high, a fact which can impair the power of the statistical comparison, especially in the presence of a weak effect, as is suggested for the aneugenic action of DEB. Concerning the main clastogenic activity of DEB, the present results are in good agreement with the literature data. Recently, Murg et al. (1999) found that DEB was effective in human lymphocytes at doses as low as 2.5 µM. In our study, after a single comparison of means a statistically significant difference in MN frequencies was found only at the highest dose tested, while a linear increase in MN with dose was detected by regression analysis.

When classification of the observed MN was done on the basis of presence/absence of a centromeric region and of the number of telomeres, a proportion of MN could not be considered for this analysis, since the main nuclei were inadequately labelled. The proportion of unclassified MN did not vary among samples scored, corresponding to ~20% of total MN. We can assume therefore that this figure represents an intrinsic technical limitation of the PRINS methodology when applied to the specific cell type used in this study. It should be noted that the same PRINS approach gave a very good performance on mouse splenocytes, where the percentage of adequately labelled cells was close to 100% even in the absence of a proteolytic pretreatment (Russo et al., 1996a,b).

If the frequency of MN in the treated cell population increases, the frequency of unclassifiable MN relative to the total number of cells scored is also expected to increase (e.g. on the expectation of 20% poor quality cells, 20 MN observed on 1000 cells versus a baseline of 10/1000 should include four unclassifiable MN versus two in the control sample). In conclusion, there is no biological meaning to the observed increase in unclassifiable MN in treated cultures.

Double labelling of telomeric and centromeric sequences allowed a very fine discrimination of the content of MN. Most of the observed C+ MN carried at least two telomeres, as

### Table V. Evaluation of DNA synthesis in the main nuclei and in MN of mono- and binucleated C6 cells, as detected by 5-BrdU labelling during the last 2 h before harvesting

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mononucleated cells</th>
<th>Binucleated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Labelled (%)</td>
</tr>
<tr>
<td>Control (matched to colcemid)</td>
<td>752</td>
<td>153 (20.4%)</td>
</tr>
<tr>
<td>Colcemid, 10⁻³ M</td>
<td>701</td>
<td>241b (34.4%)</td>
</tr>
<tr>
<td>Control (matched to DEB)</td>
<td>761</td>
<td>87 (11.4%)</td>
</tr>
<tr>
<td>DEB, 10⁻³ M</td>
<td>796</td>
<td>134b (16.8%)</td>
</tr>
</tbody>
</table>

+-, positive labelling; −, negative labelling.

aIncluding cells with one labelled and one unlabelled nucleus.

bP < 0.005 (G-test).

The most probable origin of these cells is apoptosis, although a direct counterstaining of cytoplasm would have been necessary to confirm this hypothesis. The results obtained here stress the importance of an adequate choice of doses to be tested for correct evaluation of the main mechanism of action of chemicals.
expected on the hypothesis that C+ MN represent whole chromosomes; the pattern observed for C− MN was also in agreement with their expected origin, as they carried only one telomere, with few exceptions. Since MN were classified only when adequate labelling was observed in the main nuclei, the presence of false negatives among C− MN should be excluded. Moreover, MN showing no labelling of the telomere sequences were seldom observed. It can be concluded that the pattern of centromeric/telomeric sequences in MN can be regarded as an accurate estimation of their origin. Among C+ MN, many of them carried three or four telomeres, indicating that a duplicated (non-disjointed) chromosome with two chromatids is contained in the MN. This observation is in full agreement with the results obtained previously on mouse splenocytes (Russo et al., 1996b) and in vitro after the spermatid MN assay (Tommassi et al., 1998). To exclude a role of DNA synthesis in the observation of MN with three or four telomeres, 5-BrdU labelling experiments were carried out in parallel with those for MN estimation. Administration of 5-BrdU during the last 2 h of culture indicated that at that time a very low proportion of binucleated cells (2%) undergo S phase (in contrast, 10−20% of mononucleated cells were labelled). Only after treatment with colcemid was the proportion of 5-BrdU-labelled binucleated cells significantly higher than the control, possibly because cells were partially synchronized after the mitotic block. Most importantly, labelled MN were never observed in binucleated cells, showing either labelled or unlabelled nuclei. In conclusion, it appears that the observed MN containing whole duplicated chromosomes do not represent a technical artifact due to DNA synthesis occurring after the formation of binucleated cells. Altogether these results lead us to conclude that, in contrast to the classical view of chromosome lagging, errors occurring before the onset of anaphase represent a common mechanism of missegregation leading to MN formation. PRINS data obtained in this study confirm the value of molecular cytogenetics for a better characterization of the mechanism of action of genotoxic agents when assayed by means of MN tests.

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References


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