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Effect of cytochalasin B on the induction of chromosome missegregation by colchicine at low concentrations in human lymphocytes

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The aim of the present work was to investigate the possible interference of cytochalasin B (cyt B) with low concentration treatment with colchicine in the induction of chromosome/chromatid loss and micronuclei in human lymphocytes mitotically activated *in vitro*. Thus, cells from a single female donor were treated with colchicine (10 or 25 nM, from 24 h after PHA addition to fixation at 66 h) either in the presence or absence of cyt B. Single lagging chromosomes/chromatids were scored in bipolar ana-telophases and greater damage (disrupted and c-anaphases) was scored in cells at anaphase. Micronuclei were scored in the first 4000 nuclei observed in both cyt B-treated (in mononucleate and binucleate cells) and untreated cultures. With the same criterion, FISH analysis was performed on 2000 nuclei where chromosome 7 and 11 centromeric DNA probes were used in pairs. Our results showed that: (i) the frequency of laggards and of micronuclei increased with colchicine concentration but in the presence of cyt B there was a lower frequency of both (with a mean reduction of ~49%); (ii) FISH analysis showed a colchicine concentration-dependent increase in nuclei with three spots for chromosome 7; (iii) a colchicine concentration-dependent increase in tetraploid cells was observed. This increase was particularly remarkable (5-fold) in cells grown in the presence of cyt B compared with cyt B-untreated cells. The observed 'cyt B effects' can be explained if it is assumed that in cytokinesis-blocked cells there is a shorter distance between the poles. As a consequence: (i) laggards would be engulfed in the nearest daughter nucleus with a consequent lower induction of micronuclei; (ii) segregating sister chromatids in heavily impaired anaphases would not travel a sufficient distance to give rise to two daughter nuclei, leading to an increased frequency of polyploid nuclei.

Introduction

Cytochalasin B (cyt B) is a chemical agent which inhibits cytoplasmic cleavage (cytokinesis) without preventing nuclear division (karyokinesis) (Carter, 1967). As a consequence, cells that have divided once in the presence of cyt B can be easily identified by the presence of two nuclei. It is noteworthy that, under this condition, all the products of a mitosis are included in the same cell.

In recent years, the cytokinesis-block (CB) method, introduced by Fenech and Morley (1985), has been widely used in the human lymphocyte micronucleus assay because it allows restriction of the analysis to cells that have undergone one mitotic division. Furthermore, the fluorescence *in situ* hybridization technique (FISH) with chromosome-specific centromeric DNA probes (Eastmond and Pinkel, 1990) on binucleate cells has been shown to be a useful approach for studying chromosome missegregation (loss and non-disjunction), allowing recognition of micronuclei (MN) containing whole chromosomes or identification of co-migration of sister chromatids in the daughter nucleus (Zijno *et al*., 1994; Marshall *et al.*, 1996).

Based on the use of the CB method, recent work suggested that in human lymphocytes treated with low concentrations of spindle poisons, non-disjunction is induced more frequently than loss events (MN) (Marshall *et al*., 1996; Minissi *et al*., 1996; Zijno *et al*., 1996a; Sgura *et al*., 1997).

A prerequisite for such a conclusion is that cyt B treatment does not interfere with the induction of chromosome missegregation or, at least, the degree of interference should be known. Earlier studies provided contradictory results on this issue. According to these investigations, cyt B does not interfere with MN induction (Fenech and Morley, 1985; Prosser *et al*., 1988), but rather reduces MN induction in combined treatments with colchicine (Antoccia *et al*., 1993) or changes the balance between MN containing chromosome fragments and whole chromosomes (Norppa *et al*., 1993; Surrallés *et al*., 1996; Falck *et al*., 1997).

The aim of the present work was to investigate the possible interference of cyt B with the induction of chromosome/chromatid loss and MN by colchicine (10 and 25 nM) in cultured human lymphocytes *in vitro*. Such colchicine concentrations were chosen because single chromosome mis-distributions are induced and the c-mitotic effect does not prevail (Gustavino *et al*., 1994). For our purposes, ana-telophases of cells grown in the presence or absence of cyt B were analysed for lagging chromosomes and chromatids, which are the events leading to formation of MN. To obtain a more general picture of the interference of cyt B with colchicine-induced spindle damage, the ratio between disrupted anaphases (in which two scattered groups of chromatids directed towards the two poles can be distinguished) and c-anaphases (in which only one group of chromatids can be distinguished) was investigated. Moreover, the MN frequency per nucleus and the distribution of hybridization centromeric signals (chromosomes 7 and 11) per nucleus were investigated in interphase cells grown in the presence or absence of cyt B. For this purpose, scoring was performed on randomly chosen activated interphase nuclei (morphologically recognizable), regardless of whether they were in mononucleate or binucleate cells. This scoring criterion was chosen because in cyt B-untreated culture it is impossible to distinguish interphase nuclei belonging to cells that have undergone mitotic division, which are comparable with binucleate cells. Using this approach, it became possible to compare randomized populations of activated nuclei

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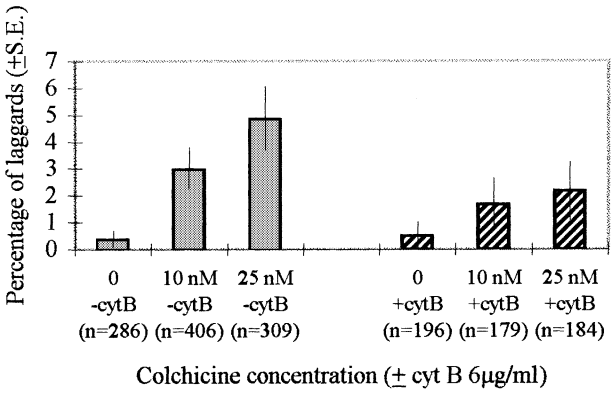


Fig. 1. Frequency (%) of single lagging chromosomes and chromatids in bipolar ana-telophases of human lymphocytes treated *in vitro* with low concentrations of colchicine in the presence or absence of cyt B. The number of ana-telophases scored is shown in parenthesis below each column. The difference in the frequency of colchicine-induced laggards between cells treated and untreated with cyt B is significant at 0.05 (χ^2 = 4.03). Data for statistical analysis were obtained by subtracting the respective control level from the two concentrations of colchicine and pooling the resulting data.

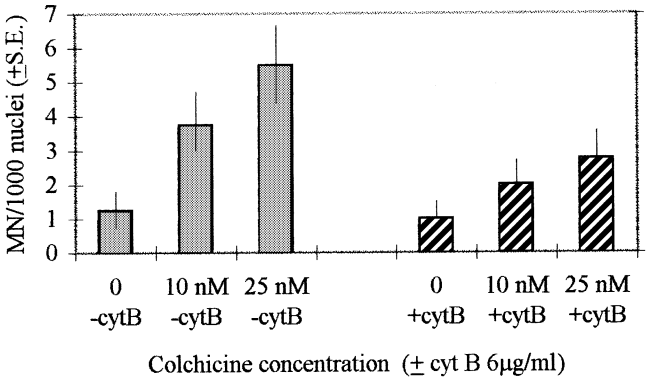


Fig. 2. MN frequency at interphase in human lymphocytes treated *in vitro* with low concentrations of colchicine in the presence and absence of cyt B. The frequency is expressed per 1000 nuclei. At each concentration 4000 nuclei were scored. In cultures containing cyt B no distinction was made between mononucleate and binucleate cells. The difference in the frequency of colchicine-induced MN between cells treated and untreated with cyt B is significant at 0.01 (χ^2 = 6.77). Data for statistical analysis were obtained by subtracting the respective control level from the two concentrations of colchicine and pooling the resulting data.

belonging to cells that had divided or not. To verify the possible interference of cyt B with progression of the cell cycle, 5-BrUdR was supplied and the frequency of M_1, M_2 and M_{3+} metaphases was evaluated.

Materials and methods

Cell culture, chemical treatment and cell fixation

Lymphocyte cultures were established with blood collected from a female donor aged 35. For each culture, 0.5 ml of whole blood was added to 4.5 ml of RPMI-1640 medium (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (Hyclone), 2% phytohaemagglutinin (Murex), 1% L-glutamine (Gibco), antibiotics (100 IU/ml penicillin and 0.1 mg/ml streptomicin) (Gibco) and 30 μM 5-BrUdR and incubated at 37°C.

Colchicine (10 or 25 nM) was added to the cultures 24 h after PHA addition and was present until fixation. In order to obtain cytokinesis-blocked cells, 44 h after PHA stimulation cytochalasin B (Sigma) dissolved in DMSO was added to a final concentration of 6 μg /ml. Three cultures were made in parallel for each concentration.

Both cyt B-treated and untreated cells were harvested 66 h after PHA stimulation. Lymphocytes were pelleted by centrifugation. After a mild hypotonic treatment (75 mM KCl for 2 min at room temperature) to preserve the cytoplasm, cells were gently fixed four times with methanol:acetic acid (3:1 for cyt B-untreated cultures and 5:1 in the case of cyt-B treated cultures). Fixed cells were stored at –20°C.

Giemsa stained slides

Slides for the analysis of anaphases and MN were stained with Giemsa (3% for 5–8 min). For each experimental point, the degree of spindle damage was studied scoring 100–200 anaphases for the frequency of normal bipolar, disrupted and c-anaphases; at least 100 bipolar ana-telophases were analysed for single lagging chromosomes and chromatids.

One hundred randomly chosen, activated cells in interphase were preliminarly scored for each point to estimate the frequencies of binucleate and multinucleate cells.

Micronucleus frequency was investigated, at each concentration, in the first encountered 4000, randomly chosen, activated interphase nuclei (morphologically recognizable) in both cyt B-treated (where nuclei were scored regardless of whether they belonged to mononucleate or binucleate cells) and cyt B-untreated cultures. MN were identified following the standard criteria (Heddle *et al*., 1983). As recommended by different authors, cyt B-induced multinucleate cells were not scored because they show a high micronucleus frequency and derive from multipolar divisions which frequently show aberrations in anaphase of cells that have divided more than once in the presence of cyt B (Lindholm *et al*., 1991; Norppa *et al*., 1993).

Slides were coded and scored blind by two scorers. Each scorer analysed half of the scored cells.

Fluorescence in situ *hybridization (FISH)*

FISH was performed using commercial centromeric DNA probes (Oncor) specific for the alphoid sequences of chromosome 7 (biotin-conjugated probe) and chromosome 11 (digoxigenin-conjugated probe). Chromosomes 7 and 11 were probed in pairs using FITC and rhodamine as fluorescent labels. Slides were pretreated with pepsin (Sigma) (50 μg/ml in 0.01 N

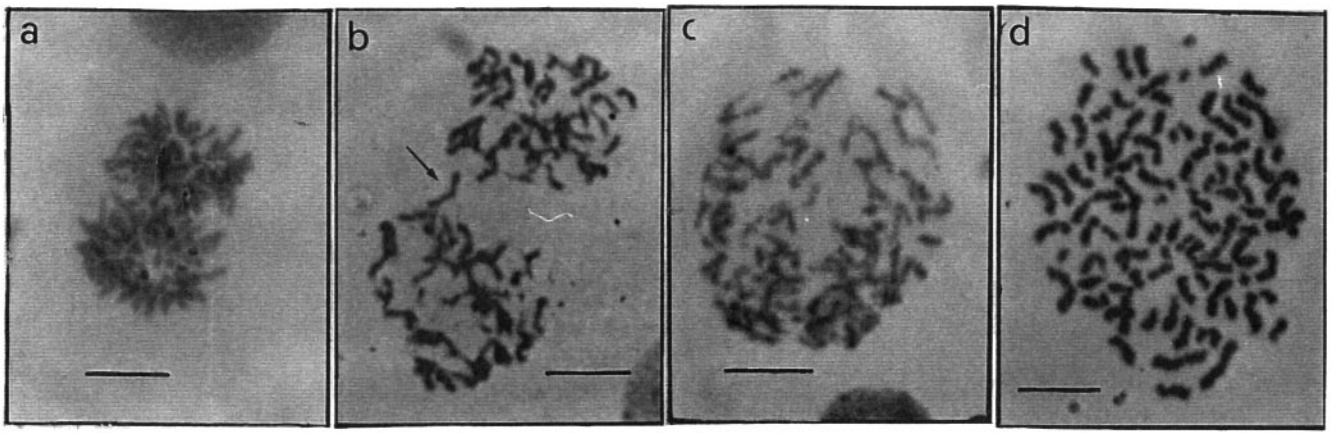


Fig. 3. Human lymphocytes treated *in vitro* with low concentrations of colchicine (10 and 25 nM). (**a**) A normal anaphase; (**b**) a lagging chromatid in a slightly disrupted anaphase (black arrow); (**c**) a disrupted anaphase; (**d**) a c-anaphase. Bars represent 10 μm.

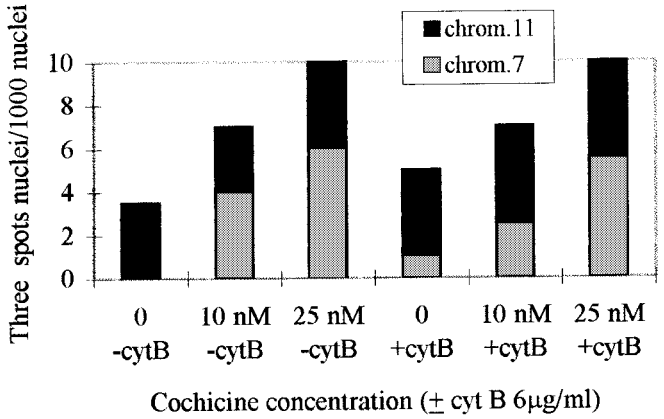


Fig. 4. Frequency of interphase nuclei with three hybridization signals for either chromosome 7 or 11 in human lymphocytes treated *in vitro* with low concentrations of colchicine in the presence and absence of cyt B. Frequency is expressed per 1000 nuclei. For each concentration 2000 nuclei were scored. In cultures containing cyt B no distinction was made between mononucleate and binucleate cells. The difference in the frequencies of nuclei with three hybridization signals for chromosome 7 between cells treated and untreated with cyt B is not significant ($\chi^2 = 1.00$, $P > 0.25$). The difference in the sum of frequencies of nuclei with three hybridization signals for either chromosome 7 or 11 between cells treated and untreated with cyt B is not significant ($\chi^2 = 1.06$, $P > 0.25$). Data for statistical analysis were obtained by subtracting the respective control level from the two concentrations of colchicine and pooling the resulting data.

HCl, 5 min at 37°C), dehydrated (3 min in cold 70, 90 and 100% ethanol) and denatured (70% formamide, 2× SSC, 2 min, at 70°C). The probes were denatured at 70°C for 5 min. Hybridization was performed overnight at 37°C in a moist chamber. The slides were washed in 50% formamide, 2× SSC at 42°C and then in 0.1× SSC at 60°C. The detection of chromosome 7 biotin-labelled probe was carried out with FITC–avidin (Oncor) and the fluorescence intensity was amplified using biotinylated anti-avidin antibody (Oncor), followed by an additional layer of FITC–avidin. The chromosome 11 digoxigenin-labelled probe was immunodetected using a mouse anti-digoxigenin antibody (Boehringer Mannheim) followed by an anti-mouse–digoxigenin antibody (Boehringer Mannheim) and anti-digoxigenin–rhodamine antibody (Boehringer Mannheim). After immunodetection slides were counterstained with DAPI (Sigma) and mounted in Vectashield (Vector laboratories).

The slides were examined with a Zeiss Axiophot microscope fitted with a FITC/rhodamine double bandpass filter set and a DAPI single bandpass filter set.

For the reasons explained above, analysis was restricted to monucleate and binucleate cells. For each experimental point, the first encountered 2000, randomly chosen nuclei were scored regardless of whether they belonged to mononucleate or binucleate cells (in cyt B-treated cultures). All nuclei with any number of hybridization signals were recorded. The frequencies of nuclei with three and four spots for one probed chromosome were estimated on all the four classes of nuclei which were found (1, 2, 3 and 4 spots). Nuclei with three hybridization signals for one chromosome always had two signals for the other. Nuclei with four hybridization signals for one chromosome always had four signals for the other, therefore they were classified as tetraploid nuclei. All the observed tetraploid nuclei were found in mononucleate cells, both in cyt B-treated and untreated cultures. Nuclei with one spot for one probed chromosome were considered mostly as artifacts due to signal overlap or poor probe penetration; their frequency ranged between 1 and 5%.

Slides were coded and scored blind by two scorers. Each scorer analysed half of the cells.

Differentially stained slides

For each experimental point, one slide was stained by the Hoechst + Giemsa technique (Perry and Wolff, 1974) and a sample of 100 metaphases was analysed for the frequency of M_1, M_2 and M_{3+} metaphases. Tetraploid metaphases were all M_2 and they were counted as two cells, being the result of a lack of segregation of the two daughter cells following anaphase failure (colchicine) or cytokinesis failure (cyt B).

Slides were coded and scored blind by two scorers. Each scorer analysed half of the scored cells.

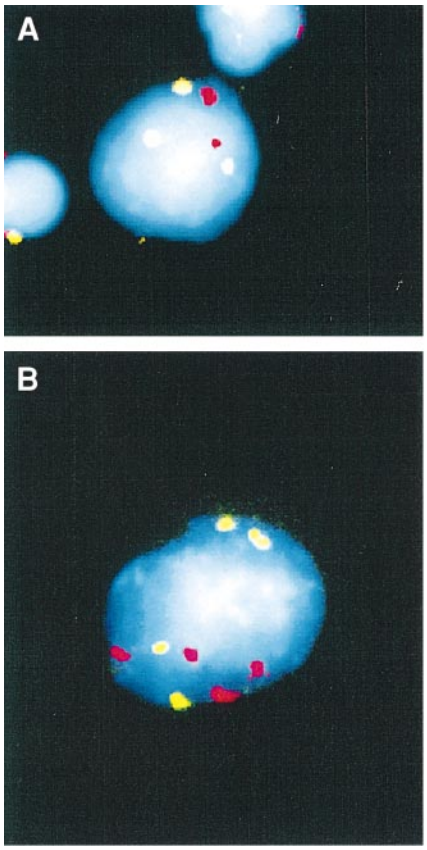


Fig. 5. Labelling of chromosome 7 (green) and 11 (red) centromeres by *in situ* hybridization in human lymphocytes treated *in vitro* with low concentrations of colchicine. (**a**) A cell with three hybridization signals for chromosome 7; (**b**) a tetraploid cell, with four hybridization signals for both chromosomes 7 and 11. Bars represent 10 μm.

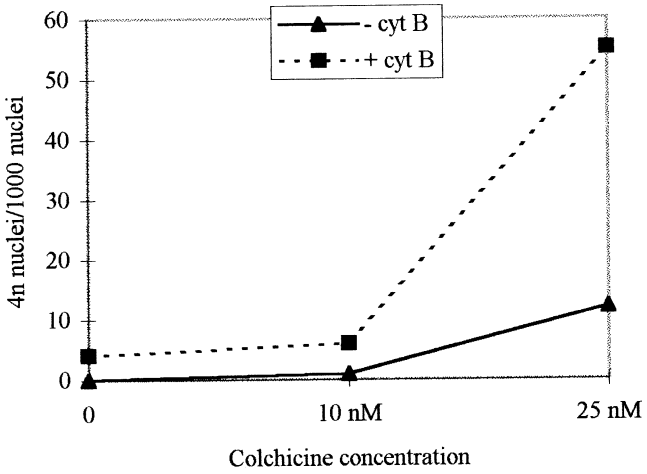


Fig. 6. The frequency of interphase nuclei with four hybridization signals for both chromosomes 7 and 11 in human lymphocytes treated *in vitro* with low concentrations of colchicine in the presence and absence of cyt B. Frequency is expressed per 1000 nuclei. At each concentration 2000 nuclei were scored; in cyt B-treated cultures nuclei were scored regardless of whether they belonged to mononucleate or binucleate cells. All scored tetraploid nuclei belonged to mononucleate cells. The difference in the frequency of colchicine-induced tetraploid cells between cultures treated and untreated with cyt B is significant at 0.001 ($\chi^2 = 50.14$). Data for statistical analysis were obtained by subtracting the respective control level from the two concentrations of colchicine and pooling the resulting data.

Statistical analysis

To compare colchicine-induced effects (chromosome/chromatid loss, MN, disrupted and c-anaphases, three spot interphase nuclei, tetraploid nuclei) between cyt B-treated and untreated cell cultures, a χ^2 test was performed. Data for statistical analysis were obtained by subtracting the respective control level from the two concentrations of colchicine and pooling the resulting data. No significant difference was observed for the above-mentioned effects in colchicine-untreated cell cultures between cyt B-treated and untreated cultures.

To compare the possible interference of cyt B with cell cycle progression for both colchicine concentrations and for colchicine-untreated cultures, χ^2 tests were performed between cultures grown in the presence and in the absence of cyt B at each colchicine concentration for the frequency of M_1, M_2 and M_{3+} cells.

Table I. Analysis of FPG stained metaphases of cells treated with colchicine in the presence (+) and absence (–) of cytochalasin B (cyt B)

Colchicine concentration (± cyt B)	M_1 (2*n*)	M_2 (2*n*)	M_2 (4*n*)	M_3 (2*n*)
0 nM –cyt B	58	42		
0 nM +cytB	57	9	17	
($\chi^2 = 0.0205$, $P > 0.75$)				
10 nM –cyt B	71	29		
10 nM +cytB	67	9	12	
($\chi^2 = 0.374$, $P > 0.5$)				
25 nM –cyt B	83	16		1
25 nM +cytB	77	9	7	
($\chi^2 = 1.125$, $P > 0.25$)				

One hundred cells were scored per experimental point considering each 4*n* metaphase as two cells.

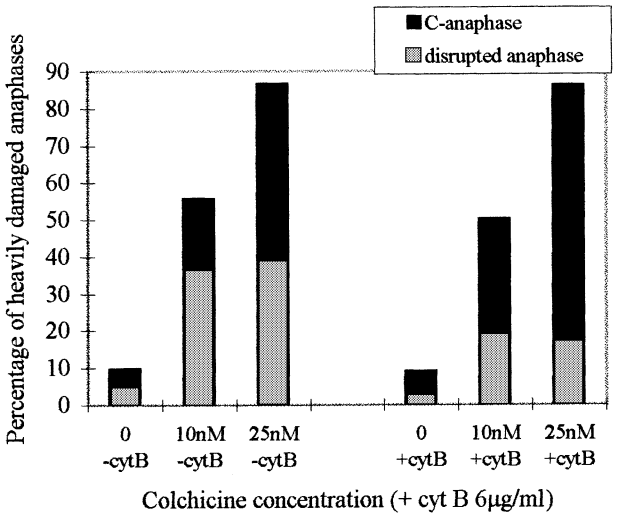


Fig. 7. Frequency (%) of disrupted and c-anaphases scored in human lymphocytes treated *in vitro* with low concentrations of colchicine in the presence and absence of cyt B. 100 anaphases were scored with cyt B and 200 anaphases without cyt B. The difference in the frequency of colchicine-induced disrupted anaphases and c-anaphases between cultures treated and untreated with cyt B is significant at 0.005 ($\chi^2 = 21.4$ and 13.86, respectively). Data for statistical analysis were obtained by subtracting the respective control level from the two concentrations of colchicine and pooling the resulting data.

Results

Binucleate cells in cyt B-treated cell cultures were 40, 40 and 35% of activated cells in cultures treated with 0, 10 and 25 nM colchicine, respectively; multinucleate interphase cells were 2, 2 and 3% of activated cells in cultures treated with 0, 10 and 25 nM colchicine, respectively; neither binucleate nor multinucleate cells were found in cyt B-untreated cultures.

The frequency of laggards in bipolar ana-telophases (Figure 1) and of MN at interphase (Figure 2) increased with colchicine concentration but in the presence of cyt B there was a lower induction of both events. Such an effect of cyt B was undetected in colchicine-untreated cultures. In colchicine-treated cultures, the mean reduction in the induction of laggards and of micronuclei was ~49% in the presence of cyt B, compared with the corresponding frequencies observed in cyt B-untreated cultures. Figure 3 shows examples of a normal anaphase (a) and of a lagging chromatid at anaphase (b) in human lymphocytes.

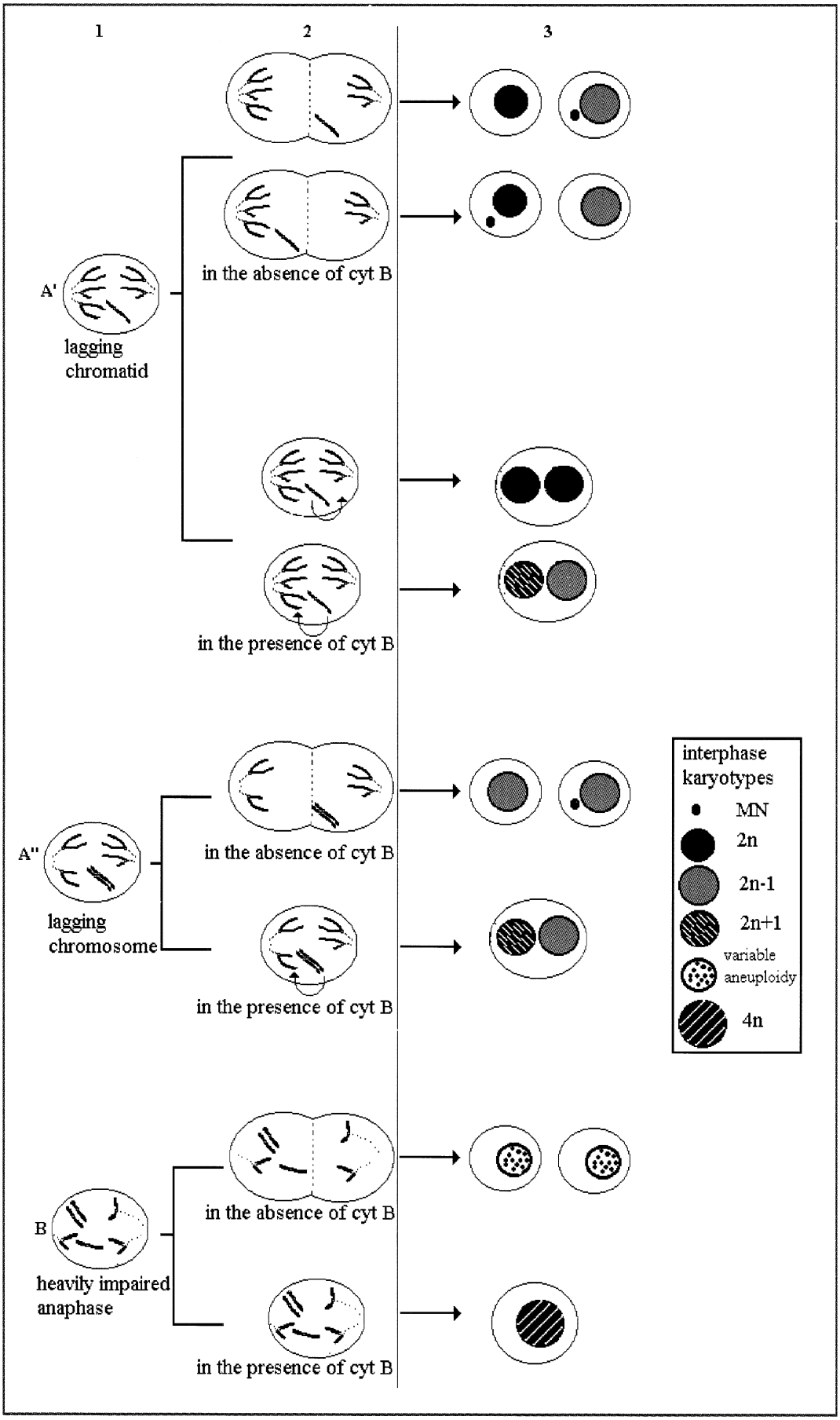
Both in the presence and in the absence of cyt B, FISH analysis showed a concentration-dependent increase in the frequency of nuclei with three hybridization signals for chromosome 7 (Figure 4). Such an increase was not observed for chromosome 11. The reason for this apparent difference is not understood at present and more studies are needed to verify it. A photograph of a human lymphocyte with three spots for chromosome 7 is shown in Figure 5. No significant difference was found for the frequencies of three spot nuclei between cyt B-treated and untreated cultures for chromosome 7.

By FISH analysis, a colchicine concentration-dependent increase in tetraploid nuclei (four well-separated signals for both chromosomes in the same nucleus; see Figure 5) was observed (Figure 6). Such an increase was particularly remarkable in cells grown in the presence of cyt B, where it was 5-fold higher than the corresponding frequency in cyt B-untreated cultures.

An increase in the frequency of induced heavy damage at anaphase (disrupted and c-anaphases) with increasing concentration of spindle poison was observed (Figure 7). Both in the presence and in the absence of cyt B a frequency of ~50% damaged anaphases was recorded at 10 nM and of ~86% at 25 nM colchicine. However, in the presence of cyt B the frequency of disrupted anaphases was about half of the corresponding value observed in the absence of cyt B, while the frequency of c-anaphases was remarkably higher. Thus, in the presence of cyt B and colchicine treatment, the most frequently induced damage is a lack of sister chromatid segregation, with a frequency of 69% of c-anaphases at the highest colchicine concentration. Figure 2 shows examples of a disrupted (c) and a c-anaphase (d).

No significant difference was found in the frequency of M_1, M_2 and M_{3+} cells between cell cultures grown in the presence and absence of cyt B in colchicine-treated (10 and 25 nM) and untreated lymphocytes (Table I). This result suggests that, independently of the colchicine treatment, the addition of 6

Fig. 8. Interference of cyt B with chromosome content in daughter cells following colchicine-induced mitotic damage. In cytokinesis-blocked cells a shorter distance between the poles is hypothesized. Following this hypothesis, laggards are engulfed in the nearest daughter nucleus with a consequent lower induction of micronuclei (A' and A'') and segregating sister chromatids in heavily impaired anaphases do not travel a sufficient distance to give place to two daughter nuclei, leading to polyploid restitution nuclei (B). The fate of a lagging chromatid (**A'**), of a lagging chromosome (**A''**) and of a heavily impaired anaphase (**B**) are shown. Black bent arrows show engulfment of a chromatid/chromosome by the main nucleus. Column 1, misdistribution events; column 2, fate of misdistributed chromosomes/chromatids at telophase in the presence and absence of cyt B; column 3, karyotype composition of interphase daughter nuclei.


1
2
3
A'
lagging chromatid
in the absence of cyt B
in the presence of cyt B
A''
lagging chromosome
in the absence of cyt B
in the presence of cyt B
B
heavily impaired anaphase
in the absence of cyt B
in the presence of cyt B
interphase karyotypes
MN
2n
2n-1
2n+1
variable aneuploidy
4n

μg /ml cyt B to the cultures does not induce any cell cycle delay up to the fixation time. M_2 tetraploid metaphases were found only in cyt B-treated cultures.

Discussion and conclusions

The lack of interference of cyt B with cell cycle progression (Table I) allows one to exclude that this is the way by which cyt B reduces the frequency of MN induced by spindle poisons.

Following our data, in mitotically activated human lymphocytes treated with low concentrations of colchicine the observed induction of MN in cyt B-treated cultures is ~49% lower than that recorded in cyt B-untreated cultures. On the basis of our estimations (computation not shown), the underevaluation of MN frequency due to the omission of scoring of multinucleate cells in cyt B-treated cultures is negligible; thus, considering such underevaluation, an estimation of the reduction in MN frequency due to the interference of cyt B is ~42%. The present data should be confirmed in replicate experiments on lymphocytes from different subjects, including male subjects.

Our results suggest that cyt B treatment interferes with chromosome missegregation in human lymphocytes treated *in vitro* with spindle poison (as first suggested by Eastmond and Tucker, 1989; Migliore *et al*., 1989). The observed 'cyt B effects' are likely to be explained if it is assumed that in cytokinesis-blocked cells the absence of the actin ring interferes with anaphase-B, leading to a shorter distance between the poles, as suggested by Norppa *et al.* (1993), Surrallés *et al.* (1996) and Falck *et al.* (1997) and measured by Cimini *et al.* (1997) in human fibroblasts. In Figure 8 the process and the consequences of a shorter pole distance on daughter nuclei are described.

According to this hypothesis laggards would be engulfed in the nearest daughter nucleus, with a consequent lower micronucleus yield and an underestimation of the frequency of loss events in balance with non-disjunction (Figure 8A′ and A′′). A similar phenomenon could be hypothesized to explain the lower frequency of MN containing whole autosomes (Surrallés *et al.*, 1996) and acentric fragments (Falck *et al.*, 1997) in binucleate human lymphocytes.

On the basis of our estimations, starting from data on laggard and MN frequency (see Appendix 1), a very small increase in three spot cell frequency, due to the engulfment of laggards into daughter nuclei, is to be expected. This explains our results on three spot cells.

Another consequence of this hypothesis is that segregating sister chromatids in heavily impaired anaphases would not travel a sufficient distance to give rise to two daughter nuclei. This may explain the transformation of disrupted anaphases into c-anaphases and, consequently, the increased frequency of polyploid nuclei we observed (Figure 8B). An increased frequency of tetraploid cells was observed by Zijno *et al.* (1996b) in human lymphocytes treated with another spindle poison, vinblastin, in the presence of cyt B. The authors proposed a similar model to explain such an effect: the lack of a cleavage furrow could favour accidental nuclear fusion after a badly damaged mitosis.

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Appendix 1. Calculation of the expected increase in the frequency of nuclei with three hybridization signals due to engulfment of lagging chromatids/chromosomes in cyt B-treated cultures

The expected relative frequency of nuclei with three hybridization signals due to engulfment of lagging chromatids/chromosomes in binucleate cells after cyt B treatment can be easily calculated on the basis of the hypothesis delineated in Figure 8A' and A''. The equation is derived by multiplying

the probabilities of single events leading to the class of cell of interest and by adding together the different probabilities of pathways leading to it. The following assumptions were made:

(i) all chromosomes act similarly, i.e. they have the same probability, among them, to be lost or engulfed;
(ii) the decrease in the frequency of laggards is due to engulfment.

The following symbols are used in the equation:

cmsl, probability of chromosome loss, measured as the relative frequency of ana-telophase with a single lagging chromosome among all scored bipolar ana-telophases;
ctdl, probability of chromatid loss, measured as the relative frequency of ana-telophase with a single lagging chromatid among all scored bipolar ana-telophases;
eng, probability of a laggard being regained by a main nucleus through engulfment;
i, expected increase in the frequency of cells with three hybridization signals for one probed chromosome.

Thus, the following equation can be written for binucleate cells

$$\mathbf{i} = (0.5\ cmsl + 0.25\ ctdl)eng/23.$$

The expected increases in the frequency of cells with three hybridization signals for one probed chromosome were calculated giving the following values to the variables of the equation, on the basis of the empirical data (Figure 1).

- Control culture: $cmsl = 0\%$, $ctdl = 0.35\%$;
- 10 nM colchicine concentration: $cmsl = 1.48\%$, $ctdl = 1.48\%$;
- 25 nM colchicine concentration: $cmsl = 2.27\%$, $ctdl = 2.59\%$.

Actually, with the present data, with $eng = 0.42$, the expected increase in trisomic nuclei for one probed chromosome is, on average, 0.2/1000 at 10 nM colchicine and 0.3/1000 at 25 nM. These values, which correspond to ~5% of the observed frequency of nuclei with three hybridization signals for one probed chromosome, are too low to be significantly detected.