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

Sandra Minissi¹, Bianca Gustavino¹, Francesca Degrassi², Caterina Tanzarella³ and Marco Rizzoni¹

¹Dipartimento di Biologia, Università di Roma ‘Tor Vergata’, Viale della Ricerca Scientifica, 00133 Roma; ²Centro di Genetica Evoluzionistica del CNR, Roma and ³Dipartimento di Biologia, Università ‘Roma Tre’, Viale Guglielmo Marconi 446, 00146 Roma, Italy

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 anaphases 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 mitotic division are the events leading to formation of MN. To obtain a more general picture of the 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, anaphases of cells grown in the presence or absence of cyt B were analyzed 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 nuclei (morphologically recognizable), regardless of whether they were in mononucleate or binucleate cells. This scoring criterion was chosen because in cyt B-untreated culture inactivated 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
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 ($\chi^2 = 4.03$). 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 ($\chi^2 = 6.77$). Data for statistical analysis were obtained by subtracting the respective control level from the two concentrations of colchicine and pooling the resulting data.

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.
analysed for the frequency of M1, M2 and M3

and a sample of 100 metaphases was

treated and untreated with cyt B is not significant ($\chi^2 = 1.06, P > 0.25$). Data for statistical

with low concentrations of colchicine in the presence and absence of cyt B.

Frequency is expressed per 1000 nuclei. At each concentration 2000 nuclei

denatured at 70°C for 5 min. Hybridization was performed overnight

denatured (70% formamide, 2 min, at 70°C). The detection of

SSC at 42°C and then in 0.1X SSC at 60°C. The detection of chromosome 7

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

anti-avidin antibody (Oncor), followed by an additional layer of FITC–

were scored; in cyt B-treated cultures nuclei were scored regardless of

and mononucleate cells (in cyt B-treated cultures). All nuclei with

and untreated with cyt B. The probes

and then in 0.1X SSC at 60°C. The detection of chromosome 7

400 nuclei. All scored tetraploid nuclei were found in mononucleate cells,

whether they belonged to mononucleate or binucleate cells. All scored

were double bandpass filter set and a DAPI single bandpass

were analysed by subtracting the respective control level from the

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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.

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. 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 at 70°C for 5 min. Hybridization was performed overnight

at 37°C in a moist chamber. The slides were washed in 50% formamide,

mononucleate and binucleate cells. The difference in the frequencies of

Two concentrations of colchicine in the presence and absence of cyt B.

both chromosome 7 and 11. Bars represent 10 μm.

Slides were coded and scored blind by two scorers. Each scorer analysed

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

metaphases were all M2 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 cells.

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half of the scored cells.
**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 $\chi^2$ 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, $\chi^2$ tests were performed between cultures grown in the presence and in the absence of cyt B at each colchicine concentration for the frequency of M1, M2 and M3, cells.

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

<table>
<thead>
<tr>
<th>Colchicine concentration (± cyt B)</th>
<th>M1 (2n)</th>
<th>M2 (2n)</th>
<th>M2 (4n)</th>
<th>M3 (2n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM – cyt B</td>
<td>58</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM + cyt B</td>
<td>57</td>
<td>9</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>($\chi^2 = 0.0205$, $P &gt; 0.75$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 nM – cyt B</td>
<td>71</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 nM + cyt B</td>
<td>67</td>
<td>9</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>($\chi^2 = 0.374$, $P &gt; 0.5$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 nM – cyt B</td>
<td>83</td>
<td>16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>25 nM + cyt B</td>
<td>77</td>
<td>9</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>($\chi^2 = 1.125$, $P &gt; 0.25$)</td>
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</table>

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

### 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 M1, M2 and M3, 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

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**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.

**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 distance between the poles. A photograph of a human lymphocyte with three spots for chromosomes 7 is shown in Figure 5. No significant difference was found for the frequencies of three spot nuclei between cyt B-treated and untreated cell cultures.

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Cyt B and colchicine-induced chromosome missegregation

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. M2 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) andacentric fragments (Falck et al., 1997) in bipolars 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.

References


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Appendix 1. Calculation of the expected increase in the frequency of nuclei with three hybridization signals due to engulfment of laggard 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:

\( c_{msl} \), probability of chromosome loss, measured as the relative frequency of ana-telophase with a single lagging chromosome among all scored bipolar ana-telophases;

\( c_{tdl} \), 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

\[
 i = (0.5 c_{msl} + 0.25 c_{tdl}) eng / 23.
\]

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: \( c_{msl} = 0\% \), \( c_{tdl} = 0.35\% \);
- 10 nM colchicine concentration: \( c_{msl} = 1.48\% \), \( c_{tdl} = 1.48\% \);
- 25 nM colchicine concentration: \( c_{msl} = 2.27\% \), \( c_{tdl} = 2.59\% \).

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.