Differences in malsegregation rates obtained by scoring ana-telophases or binucleate cells

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In this work we have applied in situ hybridization with alphoid centromeric probes specific to chromosomes 7 and 11 to ana-telophase cells from human primary fibroblasts. The aim was to visualize the events leading to aneuploidy directly during ana-phase, analyse the induction of aneuploidy during this mitotic stage and compare the frequencies of chromosome malsegregation observed in ana-telophases with the estimated malsegregation obtained in binucleate cells after a short cytochalasin B treatment. Significantly higher frequencies of chromosome loss and chromosome non-disjunction were observed in fibroblasts undergoing ana-telophase during recovery from a nocodazole-induced mitotic arrest compared with binucleate cells obtained by a further 30 min incubation with cytochalasin B. Using the same experimental schedule, analysis of hybridization signals in mononucleate cells showed higher frequencies of polyploidy nuclei in cytochalasin B-treated cultures, indicating that part of the ana-telophases observed after release from the nocodazole-induced mitotic arrest may give rise to polyploid mononucleate cells instead of binucleate ones. A reduced distance between spindle poles was also measured in cells undergoing ana-telophase in the presence of cytochalasin B. Our study suggests that in nocodazole and cytochalasin B-treated cultures the shorter pole-to-pole distance may favour the reformation of a single membrane around telophase chromosomes, especially when several lagging chromosomes lie between the two future daughter nuclei. This would give rise to polyploid mononucleate cells at the ensuing interphase.

Introduction

In the last few years, the introduction of cytochalasin B (Cyt B) in the micronucleus assay, together with anti-kinetochore staining or fluorescence in situ hybridization with pan-centromeric probes, has led to a breakthrough in the analysis of micronuclei in vitro. It has allowed the analysis of both chromosome loss and breakage following treatment of mammalian cells with chemical or physical agents, restricting the analysis to cells that have passed through mitosis once after the treatment (Eastmond and Tucker, 1989; Antoccia et al., 1993; Noorpa et al., 1993). A unique feature of the method is that it enables identification of the reciprocal products of a mitosis, since both daughter nuclei are maintained in the same cytoplasm as a result of the inhibitory action of Cyt B on cytokinesis (Carter, 1967). The use of chromosome-specific centromeric probes on cytokinesis-blocked cells not only allows chromosome loss to be detected as FISH-positive micronuclei in binucleate cells, but also erroneous distribution of chromosomes between the main nuclei (non-disjunction) to be identified through analysis of the hybridization signals (Zijno et al., 1994; Boei et al., 1995; Kirsch-Volders et al., 1996). This method has been applied to different cell types and several papers have been devoted to the validation of the method as a possible test for aneuploidy-inducing agents. Advantages and disadvantages of the method have been discussed in a recent paper (Fenech, 1997). Following this approach, the aneuploidy-inducing potentials of several spindle poisons such as colchicine, vinblastine, carbendazim, mebendazole, nocodazole and vincristine sulphate (Marshall et al., 1996; Zijno et al., 1996a; Elhajouji et al., 1997; Sgura et al., 1997) have been investigated and the induction of aneuploidy in human cells after treatment with X-rays (Kirsch-Volders et al., 1996) or the topoisomerase II inhibitor etoposide (Cimini et al., 1997) has been shown. Results from several papers suggest that misdistribution of chromosomes between main nuclei occurs more frequently than chromosome loss following exposure to aneuploidy-inducing agents and that non-disjunction is induced at lower doses of spindle poisons (Kirsch-Volders et al., 1996; Marshall et al., 1996; Zijno et al., 1996a; Elhajouji et al., 1997; Sgura et al., 1997).

The usefulness of the Cyt B method is based on the assumption that interphase analysis of signal distribution in binucleate cells truly represents the distribution of chromosomes between the two spindle poles during ana-telophase or the failure to incorporate them into the main nuclei at telophase.

In this work we have applied in situ hybridization with chromosome-specific centromeric probes to ana-telophase cells from human primary fibroblasts to visualize the events leading to aneuploidy directly during ana-phase, to investigate the induction of aneuploidy during this mitotic stage and to compare the frequencies of chromosome malsegregation observed in ana-telophase with the estimated malsegregation obtained in binucleate cells. This was done following treatment with the spindle inhibitor nocodazole (NOC), as a model compound for chemicals producing mitotic arrest. Human primary fibroblasts were arrested in prometaphase in the presence of NOC. Cells were then briefly released in drug-free medium to analyse chromosome loss and non-disjunction in ana-telophase cells by means of in situ hybridization with chromosome 7- and 11-specific alphoid probes. At the end of the recovery time, parts of the cultures were further incubated with Cyt B for 30 min to compare frequencies of malsegregation in ana-telophase and binucleate cells obtained by this short Cyt B treatment. Higher frequencies of malsegregation were observed in mitotic cells than in binucleate interphases. The origin of this discrepancy was investigated by analysing the induction of polyploid mononucleate cells and the reduction of pole-to-pole distance in ana-telophase cells after Cyt B treatment.

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Cells were treated with 35 ng/ml NOC for 4 or 16 h and then released in fresh medium for 30 or 60 min. One half of the cultures were then harvested to score ana-telophases while the other half was incubated with 3 µg/ml Cyt B for a further 30 min to obtain binucleate cells.

CL, chromosome loss; ND, non-disjunction.

*DMSO concentration in the medium was 0.125%.

Pooled data for 4 and 16 h treatments.

*P < 0.01 and **P < 0.05 by the χ² test, when comparing values obtained from scoring ana-telophases with the respective values obtained from scoring binucleate cells.

### Materials and methods

#### Cell culture and treatments

Human lung diploid fibroblasts (MRC-5 cells, obtained from the American Type Culture Collection, Manassas, VA) were maintained in minimum essential medium, supplemented with 10% foetal calf serum, antibiotics and non-essential amino acids. Confluent cultures were trypsinized and cells seeded on 22x22 mm coverslips in 35 mm Petri dishes and incubated at 37°C to allow growth. Cultures were treated with 35 ng/ml NOC for 4 or 16 h to arrest cells in prometaphase or else received 0.125% DMSO, the NOC solvent, for 16 h. At the end of the treatment, cells were washed once in phosphate-buffered saline and once in complete medium and then reincubated in fresh medium in order to release cells from the mitotic block. After 30 or 60 min, half of the cultures were fixed in a 3:1 methanol:acetic acid mixture for cell frequencies were evaluated on slides obtained from cells arrested for 16 h in prometaphase by NOC treatment and then released in drug-free medium in order to release cells from the mitotic block. After 30 or 60 min, half of the cultures were fixed in a 3:1 methanol:acetic acid mixture for analysis of ana-telophase cells, while the other half was incubated with 3 µg/ml Cyt B for a further 30 min to obtain binucleate cells and fixed thereafter. Cultures receiving Cyt B for 8 h were used as controls for malsegregation incidence in binucleate cells, since pilot experiments indicated that human fibroblasts did not yield a sufficient number of binucleate cells for scoring. The frequencies of the different types of chromosome malsegregation were evaluated on these samples after FISH staining. Polyploid mononucleate cell frequencies were evaluated on slides obtained from cells arrested for 16 h in prometaphase by NOC treatment and then released in drug-free medium and Cyt B as described above. For the analysis of polyploidy induction by Cyt B alone, 3 µg/ml Cyt B were added to MRC-5 cultures and cells were fixed after different incubation times (4, 8 and 24 h) and processed for ana-telophase analysis after 30 or 60 min recovery or, alternatively, were further post-incubated with Cyt B for 30 min for the analysis of binucleate cells. This short Cyt B treatment allowed a population of binucleate cells directly comparable with ana-telophases observed 30 min earlier to be selected, since pilot experiments indicated that human fibroblasts progressed from anaphase to the next interphase in ~30 min. Coverslips were processed for FISH using 6 ng of a biotin-labelled chromosome 7 alpha probe (Oncor, Gaithersburg, MD). In situ hybridization was performed as previously described (Cimini et al., 1997). Preparations were examined on a Zeiss Axioskop microscope equipped with UV-H 365 and FITC/rodamine double bandpass filters for visualization of DAPI and hybridization signals, respectively. On each coverslip, all ana-telophase cells or all binucleate cells in Cyt B-treated cultures were visualized and the number of hybridization signals was recorded. Numbers of hybridization signals ≥4 for each probe were considered as due to poor hybridization efficiency or signal overlap (<4) or non-specific hybridization (>4). Their mean frequencies per 100 cells ± SE were 0.09 ± 0.79 and 6.15 ± 1.36 in ana-telophases and binucleate cells, respectively, when analysing the data from all scored slides. The difference between the two means was not statistically significant (t-test, t = −0.036, P = 0.972), indicating similar hybridization efficiency in interphase and mitotic cells. Only ana-telophases or binucleate cells with the correct number of hybridization signals (four FITC and four rhodamine signals per cell) were analysed for the distribution of probe signals. Ana-telophase cells were considered normal when 2 signals per chromosome were located at each pole and binucleate cells were considered normal when the signals were evenly distributed between the two daughter nuclei. When scoring binucleate cells, strictly conservative criteria were used to avoid misscoring of paired signals due to S/G2 replicated centromeres as two signals. Paired signals were recorded as a single signal unless they were separated by a span greater than the diameter of the largest signal present in the optical field; moreover, signals connected by a thin thread of DNA, even if widely separated, were always scored as a single signal. 

### Results

#### Malsegregation in ana-telophases and in binucleate cells

Human fibroblasts blocked at prometaphase by a 4 or 16 h NOC treatment were released in fresh medium and fixed for ana-telophase analysis after 30 or 60 min recovery or, alternatively, were further post-incubated with Cyt B for 30 min for the analysis of binucleate cells. This short Cyt B treatment allowed a population of binucleate cells directly comparable with ana-telophases observed 30 min earlier to be selected, since pilot experiments indicated that human fibroblasts progressed from anaphase to the next interphase in ~30 min. Coverslips were processed for FISH using chromosome 7- and 11-specific alpha probes and ana-telophase cells were analysed for the distribution of signals to the spindle poles or on lagging chromosomes (Figure 1a–c). A middistribution of signals (3 signals on one pole and 1 signal on the other pole) was considered as indicative of a non-disjunctional event (Figure 1b), whereas the presence of FISH-positive lagging signals was considered as indicative of a non-disjunctional event (Figure 1b).
chromosomes was taken as indicative of a chromosome loss event (Figure 1b and c). Using the same hybridization strategy, the distribution of hybridization signals on daughter nuclei or on micronuclei of binucleate cells was also evaluated (Figure 1d–f). Frequencies of malsegregation were analysed by pooling data from cultures allowed to recover for 30 or 60 min after NOC treatment, since the cumulative frequency of ana-telophases observed at these two recovery times was found to correspond approximately to the frequency of mitotic-arrested cells observed at the end of the NOC treatment (data not shown). Furthermore, results from the two fixation times were pooled because low absolute values of malsegregation events were recorded in our analysis, which was restricted to malsegregation involving chromosomes 7 and 11.

A significant induction of malsegregation events for chromosomes 7 and 11 was observed both in ana-telophase and binucleate cells when human fibroblasts were exposed to 35 ng/ml NOC (Table I). The incidence of total malsegregation was higher when fibroblasts were incubated in the presence of NOC for 16 h than in the case of shorter NOC treatment in both ana-telophase and binucleate cells ($P < 0.001$ in both cases, $\chi^2$ test). After both the 4 and 16 h NOC treatments no statistically significant difference in the frequencies of total malsegregation was observed between the two chromosomes investigated, either in ana-telophases or in binucleate cells (Table I).

For both treatment times, higher frequencies of both chromosome loss and non-disjunction were always observed in ana-telophase than in binucleate cells (Table I). Total malsegregation frequencies for chromosomes 7 and 11 were significantly higher in ana-telophases than in binucleate cells both after 4 h and following 16 h of NOC treatment ($P = 0.01$ in both cases). The frequency of lagging chromosomes showing hybridization signals for chromosome 7 and/or 11 among ana-telophases was statistically higher than the frequency of FISH-positive micronuclei for chromosome 7 and/or 11 in binucleate cells when the data from the two treatment times were pooled ($P < 0.05$). The same was found for non-disjunction observed in ana-telophases compared with binucleate cells ($P < 0.01$). Frequencies of malsegregations in control cultures were similar in both binucleate and anaphase cells. However, the absolute low incidence of malsegregation in the cell line investigated did not allow sound statistical comparisons between ana-telophase and binucleate cells using control data.

**Frequency of polyploid cells**

*In situ* hybridization with alphoid probes for chromosomes 7 and 11 was also used to evaluate the frequencies of polyploid cells in 16 h NOC-treated cultures at the two recovery times as well as in Cyt B post-incubated samples. Cells were considered polyploid when four widely separated, unpaired, hybridization signals for each chromosome were present on a single nucleus. Figure 2 shows that polyploid cell frequencies were higher after 30 min exposure to Cyt B in cultures recovering from the NOC-induced mitotic arrest. Mean frequencies of polyploid mononucleate cells were statistically higher after Cyt B for both the 30 and 60 min recovery times ($P < 0.01$ and $P < 0.05$, respectively, Student’s t-test).

Using the same FISH protocol, polyploid mononucleate and binucleate cell frequencies were analysed following exposure of variable duration to 3 μg/ml Cyt B. A time-dependent induction of both polyploid mononucleate and binucleate cells was observed when Cyt B was applied for 4, 8 and 24 h in human fibroblasts (Figure 3). Polyploid mononucleate cell frequencies were statistically higher after 8 or 24 h growth in the presence of the actin inhibitor ($P < 0.05$, Student’s t-test) than in control cultures. As expected, binucleate cell frequencies also increased statistically in cells receiving Cyt B for 8 or 24 h ($P < 0.05$ and $P < 0.01$, respectively). Binucleate cells were induced at higher frequencies compared with polyploid ones for all tested treatment times, confirming
earlier results that Cyt B efficiently induces binucleate cells (Carter, 1967; Kelly and Sambrook, 1973; Degrassi et al., 1993).

**Pole-to-pole distance**

In order to understand the mechanisms involved in the induction of polyploid cells by Cyt B, the distance between the spindle poles was measured in two samples of Giemsa-stained ana-telophases observed in both the absence and in the presence of Cyt B. Figure 4 illustrates the typical elongated shape displayed by ana-telophases from human fibroblast cultures (Figure 4a), whereas ana-telophases observed after 30 min exposure to Cyt B in cells recovering from NOC-induced mitotic arrest showed shrinkage and rounding up of the cytoplasm (Figure 4b). The histograms in Figure 4 report the actual measurements of the distance between the poles in a group of Cyt B-treated cells and in a group of untreated cells. A statistically significant ($P < 0.001$, Student’s $t$-test) shorter pole-to-pole distance was observed in the Cyt B-treated cell population than in untreated cells. Mean values ± SE in Cyt B-treated and untreated samples were 18.5 ± 0.4 and 25.5 ± 0.5 μm, respectively.

**Discussion**

Comparison of the incidence of chromosome 7 and 11 loss and non-disjunction between ana-telophase and binucleate cells showed that malsegregation frequencies observed in binucleate cells after NOC treatment were consistently lower than those observed in ana-telophases. No indication for such a difference was obtained from control cultures. The quantitative discrepancy observed in cells recovering from the NOC treatment can perhaps be accounted for by the fact that some of the analysed ana-telophases could not give rise to binucleate cells and, therefore, may have been excluded from the interphase scoring, which is restricted to binucleate cells only. The present study suggests that this could be the case, since a statistically significant induction of polyploid mononucleate cells was observed when prometaphase-blocked fibroblasts were released in drug-free medium for a short time and post-incubated with Cyt B for a further 30 min. In line with this result, previous studies showed that the presence of Cyt B synergistically increases the induction of polyploid nuclei by vinblastine (Zijno et al., 1996b) or colchicine (Minissi et al., 1999) when human lymphocytes are incubated with Cyt B together with the spindle poison for approximately one cell cycle.

The present study indicates that in the presence of Cyt B a fraction of ana-telophases containing lost or misdistributed chromosomes may give rise to polyploid mononucleate cells instead of binucleate ones, changing the frequencies of malsegregation observed in the two cell populations. A role in this phenomenon may be attributed to the shorter pole-to-pole distance observed in ana-telophases from cultures receiving Cyt B. These ana-telophases are characterized by absence of the actin-based contractile ring, which results in an altered cell morphology. A recent study shows that, as well as the contractile ring, the tubulin central spindle is also absent from ana-telophases from cultured mammalian cells treated with Cyt B and that the two structures strongly cooperate during cytokinesis (Cimini et al., 1998). The present study suggests that the contractile ring and central spindle cooperate not only in the completion of cytokinesis but also in distancing and separating the two daughter sets of chromosomes during ana-telophase, which suggests an interdependency between karyokinesis and cytokinesis during mitosis. Thus, it can be hypothesized that in Cyt B-treated ana-telophases from NOC-treated cultures the two groups of segregating chromosomes may remain sufficiently close together to be included in the same nucleus during nuclear envelope reformation due to the simultaneous absence of both the contractile ring and central spindle. This could be favoured in cells with several lost chromosomes lying between the two sets of segregated chromosomes. Indeed, analysis of nuclear envelope assembly in cell-free extracts (Marshall and Wilson, 1997) or time-lapse imaging of mitotic cells (Ellenberg et al., 1997) revealed that the nuclear membrane is reassembled at the end of mitosis through the binding of vesicles containing nuclear membrane proteins.
to the surface of chromatin and their successive fusion until the chromatin is enclosed in a double membrane. Therefore, it could be envisaged that the presence of chromatin between the two future daughter nuclei may direct the positioning of nuclear membrane vesicles and promote the formation of a single polyploid nucleus in abnormally segregated anatalephases.

Lower frequencies of centromere-negative or centromere-positive micronuclei have already been observed in cultures receiving Cyt B than in cultures grown without the actin inhibitor following treatment with several spindle poisons (Antoccia et al., 1993; Surrallés et al., 1996; Falck et al., 1997; Minissi et al., 1999). In several of these studies the authors hypothesized that a short distance between the spindle poles in Cyt B-treated cells may diminish the likelihood of micronucleus formation due to engulfment of micronuclei in one of the main nuclei (Surrallés et al., 1996; Falck et al., 1997; Minissi et al., 1999). However, this hypothesis could not be tested in their experimental material, i.e. human lymphocyte cultures. The present paper may be the first direct demonstration of a Cyt B-induced shorter distance between the spindle poles due to the fact that human fibroblasts grow as a monolayer and possess a well-defined cellular morphology. Our results show that both chromosome loss and non-disjunction are modified by Cyt B treatment, suggesting that, besides micronucleus engulfment, the fusion of daughter nuclei at the end of telophase may also occur in Cyt B-treated cells after NOC treatment. The two possible processes occurring at the end of mitosis, i.e. engulfment of micronuclei and formation of polyploid mononucleate cells may have different consequences on malsegregation frequencies in binucleate cells may be hard to evaluate, because it may also be modified by several factors, such as spindle poison dose and cell type. Time-lapse studies of mitotic exit of abnormally segregated telophases after spindle poisons could be used to obtain direct evidence of both phenomena.

The lower frequencies of malsegregation events observed in this study when interphases were compared with mitotic cells may have implications for the use of the cytokinesis-block method for testing purposes. However, it should be stressed that interphase analysis of binucleate cells using chromosome-specific centromeric probes has already proven to be a very sensitive method for investigating aneuploidy induction compared with other available techniques, such as metaphase counting or centromere-positive micronuclei analysis (Marshall et al., 1996; Zijno et al., 1996; Elhajouji et al., 1997). Therefore, at the present time, the in situ analysis of binucleate cells remains a very useful and sensitive method to detect aneuploidy, due also to its capacity to discriminate chromosome loss from chromosome non-disjunction. When performing such an analysis it should be kept in mind that the observed malsegregation frequencies could lead to underestimation of the effects induced in mitotic cells by spindle poisons. In this respect, the combined FISH analysis of polyploid mononucleate cells and chromosome loss/non-disjunction in binucleate cells could substantially improve the sensitivity of the cytokinesis-block assay in detecting agents affecting mitotic division.

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