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Direct and indirect non-disjunction in the origin of trisomy in cultured human lymphocytes

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The aim of the present work was to investigate the processes involved in the origin of trisomic karyotypes, i.e. co-migration of sister chromatids (mitotic non-disjunction, MND) and recovery of micronuclei (MN) originating from lagging chromosomes/chromatids at anaphase (mitotic indirect non-disjunction, MIND), and to evaluate their relative contribution to aneuploidy in human lymphocytes mitotically activated *in vitro*. Therefore, phytohaemagglutinin-stimulated human lymphocytes from one donor were treated with 10 and 25 nM colchicine and analysed through two cell cycles by means of both molecular (FISH with centromeric DNA probes specific for chromosomes 7 and 11) and classical cytogenetic techniques. The following events were analysed: (i) chromosome/chromatid loss (a MN-generating event) in M_1 bipolar ana-telophases; (ii) MN recovery in M_{2+} prophases; (iii) non-disjunction and loss of chromosomes 7 and 11 by FISH analysis in cytochalasin B-induced binucleate cells; (iv) spontaneous frequency of trisomic cells by chromosome counting and FISH analysis in M_1 c-metaphases; (v) induced frequency of trisomic cells by chromosome counting and FISH analysis in M_2 c-metaphases. Our results indicate that MND plays a major role compared with MIND in the origin of trisomic karyotypes, being ~4- to 5-fold higher in colchicine-treated cells. Moreover, remarkable reductions in the observed frequencies of trisomic cells were recorded in comparison with the expected ones, with an observed/expected frequency ratio of trisomic M_2 c-metaphases ranging between 1/3 and 1/6.

Introduction

Aneuploid karyotypes can originate at mitosis through two processes. The first is non-disjunction of sister chromatids at anaphase (mitotic 'direct' non-disjunction, MND), in which one daughter cell becomes trisomic and the other becomes monosomic. The other process involves chromosome or chromatid loss where, in the former case, both daughter cells become monosomic and, in the latter, one daughter cell becomes monosomic and the other remains normal. Generally, the lost chromosome/chromatid forms a micronucleus (MN) which is randomly distributed into one of the daughter cells. According to the mechanism of mitotic indirect non-disjunction (MIND), if the micronucleus is able to perform DNA synthesis and mitotic condensation synchronously with the main nucleus, at the following cell cycle it may be recovered into the main nucleus, giving rise to either trisomic or normal cells depending on the karyotype of the host cell (Rizzoni *et al.*, 1989; Gustavino *et al.*, 1994).

The most commonly used tests for spontaneous and chemically induced aneuploidy involve chromosome counting at metaphase, analysis of mitotic alterations, including c-mitosis, chromatid separation, chromosome lagging, multipolar spindles, cell cycle delay (Parry *et al.*, 1982; Liang and Satya-Prakash, 1985; Dulout and Natarajan, 1987), and micronucleus analysis (Heddle *et al.*, 1991), further improved by methods that allow the analysis to be restricted to cells that have divided (Pincu *et al.*, 1984; Fenech and Morley, 1985) and allow identification of MN produced by mitoclastic rather than clastogenic events (Yamamoto and Kikuchi, 1980; Degrassi and Tanzarella, 1988; Vanderkerken *et al.*, 1989). However, MND cannot be directly studied by any of these methods.

Nowadays, the application of molecular cytogenetic methods based on *in situ* hybridization (FISH) with chromosome-specific fluorescent DNA probes has made possible the direct investigation of induced and spontaneous MND (Eastmond and Pinkel, 1990). Furthermore, when FISH with chromosome-specific centromeric DNA probes is applied to cytokinesis blocked (CB) cells (Fenech and Morley, 1985), it is possible to follow the reciprocal products of chromosome (mis)distribution at mitosis in daughter interphase nuclei and MN in the same cell membrane (Zijno *et al.*, 1994; Marshall *et al.*, 1996). By means of this method (centromeric FISH on CB cells), recent studies have pointed out that in CB human lymphocytes exposed to low concentrations of spindle poisons, MND is the prevalent induced chromosome misdistribution (Marshall *et al.*, 1996; Elhajouji *et al.*, 1997; Sgura *et al.*, 1997).

In our previous studies we provided evidence for the occurrence of MIND but we could not investigate MND directly (Rizzoni *et al.*, 1989; Gustavino *et al.*, 1994). In the present study we aim to investigate both MND (co-migration of sister chromatids) and MIND (recovery of MN) in order to achieve a deeper understanding of the processes involved in the production of trisomic karyotypes and to evaluate their relative contributions to aneuploidy in human lymphocytes treated *in vitro* with low concentrations of colchicine. Therefore, phytohaemagglutinin (PHA)-stimulated human lymphocytes were treated *in vitro* with the same colchicine concentrations as in our previous study (Gustavino *et al.*, 1994). FISH with alphoid DNA probes for chromosome 7 and 11 was performed on CB cells to provide an estimation of MND. Spontaneous and induced frequencies of trisomic cells were evaluated by FISH analysis in M_1 and M_2 c-metaphases, respectively, recognizable after fluorescence sister chromatid differentiation (Kulka *et al.*, 1995). Moreover, FPG stained slides (Perry and Wolff, 1974) were scored for: (i) lagging chromosomes/chromatids in M_1 bipolar ana-telophases; (ii) state of condensation of MN in micronucleated M_{2+}; (iii) frequency of trisomic M_1 and M_2 c-metaphases.

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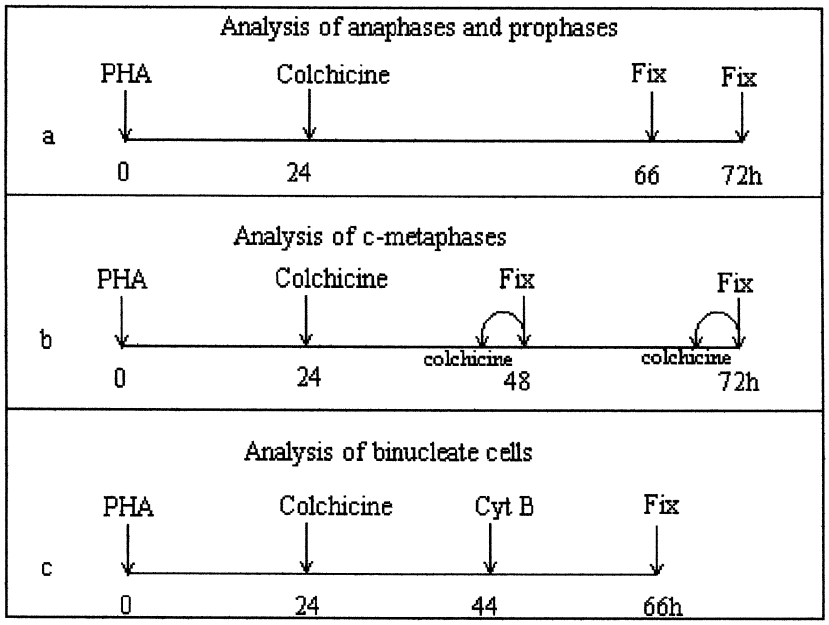

Fig. 1. Experimental schedule.

The observed frequencies of trisomic M_2 c-metaphases, evaluated in FPG stained cells, were compared with the expected frequencies calculated on the basis of data on laggards (chromosome/chromatid loss), condensed MN and MND in binucleate cells. However, since it has been demonstrated that in the presence of cytochalasin B (cyt B) the frequency of colchicine-induced lagging chromosomes/chromatids and of MN in human lymphocyte cultures is ~50% lower than in the absence of cyt B, possibly due to engulfment of laggards by the nearest daughter nucleus (Minissi *et al.*, 1999), data on MND were corrected accordingly.

Our results show that the contribution of MND to the origin of trisomic karyotypes is 3- to 5-fold higher than that of MIND in human lymphocytes treated *in vitro* with low concentrations of colchicine. Moreover, a ratio between 1/3 and 1/6 of observed versus expected frequency of trisomic M_2 c-metaphases was recorded, indicating a strong reduction in trisomic cells in the M_2 population.

Materials and methods

Cell culture, chemical treatment and cell fixation

Lymphocyte cultures were established with blood collected from a healthy female donor aged 35. The donor was chosen on the basis of a preliminary cytogenetic analysis, which revealed a very low spontaneous frequency of both chromosomal aberrations and mitotic anomalies.

For each culture, 0.5 ml of whole blood were added to 4.5 ml of RPMI-1640 medium (Sigma) supplemented with 20% heat-inactivated fetal bovine serum (Hyclone), 2% PHA (Murex), 1% L-glutamine (Gibco), antibiotics (100 IU penicillin/ml and 0.1 mg/ml streptomycin) (Gibco) and 30 μM 5-BrUdR and incubated at 37°C.

Colchicine (10 and 25 nM) was added to the cultures 24 h after PHA addition and was present until fixation. In a previous work it was demonstrated that such concentrations are most suitable to induce single chromosome misdistributions in human lymphocytes (Gustavino *et al.*, 1994).

In order to obtain binucleate cells, 44 h after PHA stimulation cyt B (Sigma) was added at a final concentration of 6 μg/ml to part of the established cultures. Triplicate cultures were made for each experimental point.

According to the experimental schedule in Figure 1a, harvesting times were chosen in order to analyse: (i) spontaneous trisomic frequency values in M_1 cells (at 48 h); (ii) MN-generating events, i.e. anaphase chromosome and chromatid laggards (at 66 and 72 h); (iii) direct non-disjunction, i.e. MND (at 66 h); (iv) MN condensation in M_2 prophase cells (at 66 and 72 h); (v) M_2 trisomic cells (at 72 h). Cultures were fixed according to the air drying method (Moorhead *et al.*, 1960) after a mild hypotonic treatment (75 mM KCl for 2 min at room temperature) to preserve the cytoplasm.

In order to obtain c-metaphase cells, half of the cultures of each set were treated with 0.5 μM colchicine 2 h before fixation at 48 and 72 h from the onset of culture (Figure 1b). In this case, hypotonic treatment was 75 mM KCl for 12 min at 37°C.

Cyt B-treated cultures for the analysis of binucleate cells were harvested 66 h from the onset of culture, to avoid scoring second generation binucleated cells (Sgura *et al.*, 1997; Figure 1c). 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 (5:1) according to the protocol developed by our group for MN analysis in binucleate cells (Surrallés *et al.*, 1992). Fixed cells were stored at –20°C.

FPG stained slides: analysis of micronuclei in prophases, laggards in ana-telophases and chromosome counting in c-metaphases

Slides were FPG stained (Perry and Wolff, 1974) so that c-metaphases could be distinguished as M_1, M_2 or M_{3+} (M_{3+} are cells which have performed at least three mitotic cycles after PHA stimulation), while ana-telophases and prophases could be distinguished only as M_1 and M_{2+}.

For each experimental point at least 500 M_1 bipolar ana-telophases were analysed for lagging chromosomes/chromatids (cells fixed at 66 h), at least 200 M_{2+} prophases were scored to detect single MN with or without synchronous mitotic condensation (cells fixed at 72 h) and 150 (cells fixed at 48 h) and 300 (cells fixed at 72 h) M_1 c-metaphases and 600 M_2 c-metaphases (cells fixed at 72 h) were scored for chromosome number. Only c-metaphases containing at least 40 chromosomes were scored.

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

FISH analysis: interphase nuclei and c-metaphases

FISH was performed using commercial centromeric DNA probes (Oncor) specific for the alphoid sequences of chromosomes 7 (biotin-conjugated probe) and 11 (digoxigenin-conjugated probe). Chromosomes 7 and 11 were probed simultaneously using FITC and rhodamine as fluorescent labels. For metaphase analysis, combined differential sister chromatid staining and centromeric FISH was carried out according to Kulka *et al.* (1995) with some modifications. Briefly, prior to FISH, slides were stained with 1 μg/ml bisbenzimide (Hoechst 33258; Sigma) at 20°C for 15 min in the dark. Subsequently, slides covered with a thin layer of the same Hoechst solution were exposed for 30 min to UV light (Osram no. L36W/73) at 20 cm distance. Slides were then washed in phosphate-buffered saline (PBS), dehydrated in 70, 90 and 100% cold ethanol and dried for 30 min at 65°C. Starting from this point, FISH was carried out as follows for both interphase and metaphase analysis.

Slides were pretreated with pepsin (Sigma) (50 μg/ml in 0.01 N 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

Table I. Percentage frequency of single lagging chromosomes/chromatids in M_1 bipolar ana-telophases and of single micronuclei (MN), with or without synchronous mitotic condensation, in M_{2+} prophases of cultured human lymphocytes treated with colchicine

Colchicine concn	M_1 ana-telophases				M_{2+} prophases			
	Scored cells	With lagging chromatid	With lagging chromosome	Total laggards	Scored cells	With condensed MN	With uncondensed MN	Total MN
0	550	0.36	0	0.36	247	0.40	0	0.40
10 nM	725	1.52	1.38	2.90[a]	351	2.28	0.28	2.56
25 nM	535	2.62	2.43	5.05[a]	344	2.62	1.16	3.78[b]

Colchicine-treated and untreated cell populations were compared by Fisher's exact test.
[a]$P < 0.01$.
[b]$P < 0.05$.

for 5 min. Hybridization was performed overnight at 37°C in a moist chamber. Post-hybridization washing consisted of three washes of the slides for 5 min each in 50% formamide, 2× SSC at 42°C, followed by three washes of 5 min each in 0.1× SSC at 60°C. Detection of the biotin-labelled chromosome 7 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 digoxigenin-labelled chromosome 11 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 (0.2 μg/ml) 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 each experimental point, 1500 binucleate cells (cyt B-treated) with well-preserved cytoplasm were scored. To evaluate the distribution of mono-, bi- and multinucleate cells, 200 cells per experimental point were preliminarily scored.

The large majority of binucleate cells was expected to have a total number of four signals for each probed chromosome. Among the cells with four signals: normal (diploid) cells were expected to contain two signals for each chromosome in each nucleus (2/2); a cell showing a trisomic and a monosomic nucleus (3/1) was expected to be due to MND; a loss event was expected to give rise to a diploid and a monosomic nucleus (2/1) with a FISH-positive MN. Cells with an odd number of signals (1/2 and 2/3) were considered to be artefacts. In order to investigate a possible bias in the analysis of the class of interest (3/1), frequencies of cells due to artefacts were used to estimate the burden of artefacts in originating the class of interest. Such a probability was calculated as follows: on pooled data, the mean frequency of 2/1 cells was assumed to be representative of the probability of a 'minus' artefact [inefficiency of probe penetration or overlap of hybridization signals (Eastmond and Pinkel, 1990)]. The mean frequency of 3/2 cells was assumed to be representative of the probability of a 'plus' artefact [splitting of the hybridization region (Eastmond and Pinkel, 1990) or non-specific hybridization]. Therefore, the probability of obtaining 3/1 cells due to double independent artefacts was estimated by multiplying 'minus' by 'plus' events. With the present data, the estimated burden of artefacts in originating the class of interest (3/1 cells) was 0.053 and 0.047% for chromosomes 7 and 11, respectively.

For each experimental point, at least 500 M_1 and 500 M_2 c-metaphases (cells fixed at 72 h) with not less than 40 chromosomes were scored.

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

Statistical analysis

For statistical analysis of the data the χ^2 and Fisher's exact test were performed in order to evaluate the differences between: the frequency of chromosome/chromatid loss, MN and trisomic c-metaphases in control and colchicine-treated cultures; the frequency of hybridization signals observed for chromosomes 7 and 11; expected and observed frequencies of trisomic M_2 c-metaphases.

Results

Analysis of lagging chromosomes/chromatids in bipolar M_1 ana-telophases

The frequencies of single lagging chromosomes/chromatids in M_1 bipolar ana-telophases obtained scoring FPG stained slides at 66 h culture time in lymphocytes from one donor are reported in Table I. A significant increase in the frequency of laggards was observed following treatment with colchicine being 0.36% in the control culture, 2.90% at 10 nM and 5.05% at 25 nM colchicine. The ratio between the relative frequencies of a single lagging chromosome and a single lagging chromatid was close to 1:1. Similar results were obtained previously (Gustavino *et al.*, 1994).

Table II. Percentage frequency of trisomic, hyperdiploid and tetraploid M_1 and M_2 FPG stained c-metaphases of cultured human lymphocytes treated with colchicine

Colchicine concn	Mitosis	Scored cells	$2n + 1$	$2n + 1 < x < 4n$	$4n$
0	M_1[a]	150	2	0.67	0
	M_1[b]	300	0.67	0	0
	M_2[b]	600	0.33	0	0.33
10 nM	M_1[a]	150	1.33	0	0
	M_1[b]	300	0.67	0	0
	M_2[b]	600	1.83[c]	0	0
25 nM	M_1[a]	150	1.33	0	0
	M_1[b]	300	0.33	0	0
	M_2[b]	600	1.83[c]	0.17	0.33

Colchicine-treated and untreated M_2 trisomic c-metaphases were compared by Fisher's exact test.
M_1 trisomic c-metaphases at 48 and 72 h were compared by Fisher's exact test, regardless of colchicine treatment.
[a]48 h cultures.
[b]72 h cultures.
[c]$P < 0.025$.

Analysis of micronuclei in M_{2+} prophases

Data about the analysis of M_{2+} prophases are also summarized in Table I. MN frequency significantly increased as colchicine concentration increased, being 0.40, 2.56 and 3.78% in control, 10 nM and 25 nM colchicine, respectively. Pooling data from cultures treated with both colchicine concentrations, 77% of single MN in M_{2+} prophase cells were in synchronous mitotic condensation with the main nucleus. Such a high probability of MN recovery is in accordance with that observed in our previous work, where 80% of MN induced in human lymphocytes by colcemid treatment was in synchronous mitotic condensation (Gustavino *et al.*, 1994). No premature chromatin condensation of MN (MN-PCC) was found.

Analysis of c-metaphases

The spontaneous frequency of trisomic cells was estimated by chromosome counting in M_1 c-metaphases in 48 and 72 h cultures (FPG stained slides) (Table II). As expected, no statistically significant difference in trisomic cell frequency was observed either in relationship to colchicine concentration or in relationship to fixing time (Fisher's exact test, $P = 0.77$)

Table III. Percentage frequency of cells containing one, two, three or four hybridization signals in c-metaphases of 72 h cultured human lymphocytes using centromeric DNA probes for chromosomes 7 and 11

Colchicine concn	Mitosis	Scored cells	Number of hybridization signals[a]							
			Chromosome 7				Chromosome 11			
			1	2	3	4	1	2	3	4
0, 10 and 25 nM	M_1[b]	1556	1.22	98.53	0.06	0.19	4.43	95.37	0	0.19
0	M_2	526	3.23	96.77	0	0	1.52	98.48	0	0
10 nM	M_2	512	3.125	96.875	0	0	4.88	95.12	0	0
25 nM	M_2	556	0.89	98.56	0	0.54	4.32	95.14	0	0.54

[a]Cells showing one or three spots for one of the probed chromosomes always had two spots for the other probed chromosome. Cells showing four spots for one probed chromosome always had four spots also for the other probed chromosome: they were polyploid cells.
[b]Data from different colchicine concentrations were pooled since, as expected, no significant difference was observed in relation to colchicine concentration.

Table IV. Distribution of hybridization signals in binucleate cells of 66 h cultured human lymphocytes using centromeric DNA probes for chromosomes 7 and 11

Colchicine concn	Type of signal distribution[a]											
	Chromosome 7						Chromosome 11					
	3/1	2/1 + MN[b]	1/1 + MN[c]	3/3	2/1[d]	3/2[d]	3/1	2/1 + MN[b]	1/1 + MN[c]	3/3	2/1[d]	3/2[d]
0	2.67	0	0	0	68.00	5.33	1.33	0	0	0.67	70.00	9.33
10 nM	3.33	0	0	0.67	64.00	6.67	4.00	0	0.67	0	47.33	8.67
25 nM	10.00[e]	0.70	0	0.67	69.33	11.33	4.67	0	0	0.67	60.00	6.00

Frequencies are expressed per thousand binucleate cells. For each experimental point 1500 cells were scored. Colchicine-treated and untreated 3/1 cells were compared by the χ^2 test.
[a]The numbers separated by the slash indicate the number of spots detected on each nucleus of a binucleate cell; MN indicates a FISH-positive micronucleus in a binucleate cell.
[b]With one spot.
[c]With two spots.
[d]Data used when calculating burden of artefacts as explained in Materials and methods.
[e]$P < 0.025$.

among the M_1 c-metaphases. Thus, at the same fixing time data were pooled and the mean value of the mean frequencies observed at the two sampling times was assumed to be representative of the spontaneous frequency of trisomy in M_1 c-metaphases. This value was 1.06%. Trisomy in M_1 c-metaphases was also studied by FISH analysis for the presence of three hybridization signals of chromosome 7 or 11. On pooled data, only one of the 1556 screened cells showed three signals for one of the probed chromosomes (chromosome 7), thus the estimated value for any chromosome is 0.74% (Table III).

The colchicine-induced frequency of trisomic cells was estimated by chromosome counting in M_2 c-metaphases in 72 h cultures (FPG stained slides) (Table II). A significant increase in the relative frequency of trisomic M_2 cells was evident in colchicine-treated cultures compared with untreated ones.

M_2 c-metaphases were also studied by FISH analysis for the presence of three hybridization signals of either chromosome 7 or 11. None of the scored cells showed three spots (Table III).

FISH analysis of interphase nuclei

The results of *in situ* hybridization on binucleate lymphocytes with chromosome 7 and 11 centromeric probes are shown in Table IV. The frequencies of spot distribution in daughter nuclei and MN are expressed per thousand cells.

Binucleate cells were 42, 40 and 36% of activated cells in cultures treated with 0, 10 and 25 nM colchicine, respectively; multinucleate (tri- and tetranucleate) cells were 2, 3 and 2% of activated cells in cultures treated with 0, 10 and 25 nM colchicine, respectively. The use of centromeric probes for chromosomes 7 and 11 provided evidence for a significant increase in the frequency of induced MND (3/1) for chromosome 7 in cells treated with 25 nM colchicine compared with control cultures (Table IV). For both the colchicine concentrations, no significant difference in the increase in frequency of 3/1 cells compared with the untreated controls was observed between the probed chromosomes.

Furthermore, FISH analysis showed the presence of binucleate cells with 3/3 spot distributions. These cells were likely to originate from trisomic circulating lymphocytes that underwent mitosis (Zijno *et al.*, 1996). Thus, the mean frequency of 3/3 binucleate cells was calculated on pooled data to estimate the spontaneous frequency of trisomy, for probed chromosomes, measured after the first cell division. Both probed chromosomes had a frequency of 0.44 per 1000 cells for spontaneous trisomy.

Discussion

The present experiments were designed to provide information about the different mechanisms (MND and MIND) involved in the origin of trisomic karyotypes in human lymphocytes treated *in vitro* with low concentrations of colchicine. Spontaneous and induced chromosome missegregation was evaluated at different cytogenetic levels: (i) chromosome

loss, directly measured by the analysis of lagging chromosomes/chromatids at ana-telophase in M_1 (MN-generating event); (ii) recovery of MN at the following prophase, which may be the origin of MIND, directly measured by the frequency of MN synchronous condensation; (iii) co-segregation of sister chromatids in binucleate cells (MND); (iv) spontaneous frequency of trisomic M_1 c-metaphases; (v) induced frequency of trisomic M_2 c-metaphases.

The spontaneous frequency of trisomic cells was estimated based on the results of both FISH analysis and chromosome counting. The frequency estimated by chromosome counting in FPG stained M_1 c-metaphases was 1.06%. The spontaneous frequency of trisomic cells estimated by FISH analysis for chromosomes 7 and 11 in M_1 c-metaphases was 0.74%. The spontaneous frequency of trisomic cells estimated by FISH analysis for chromosomes 7 and 11 in 3/3 binucleate cells was 1.02%. Estimation based on the results of FISH analysis can be obtained by multiplying the mean frequency, between the probed chromosomes, of the class of interest by 23, assuming that all chromosomes behave similarly. With the exception of sex chromosomes, this is fairly true, as evidenced by Marshall *et al.* (1996) by FISH probing for six different chromosomes (1, 8, 11, 17, 18 and X) in binucleate cells after aneuploidy induction with spindle poisons. Our results further support this approach, since the different estimations of the spontaneous frequency of trisomy obtained are very similar.

The expected relative frequencies of trisomic cells at the first mitosis after misdistribution at each colchicine concentration were calculated on the basis of the model and the assumptions described in Rizzoni *et al.* (1989), with some modifications due to the introduction of molecular cytogenetic analysis (FISH). The following equation was used:

$$f(2n + 1) = syc(0.5\ cmsl + 0.25\ ctdl) + 23(0.5\ nd_{(7-11)}) + cnt$$

- $f(2n + 1)$ is the expected relative frequency of trisomic cells among all the c-metaphases at the first mitosis after misdistribution.
- *cmsl* and *ctdl* are the sum of the probabilities of chromosome (*cmls*) and chromatid (*ctdl*) losses for any chromosome, measured as the relative frequency of ana-telophases with a single lagging chromosome or chromatid among all the M_1 ana-telophases.
- *syc* is the mean probability of synchronous mitotic condensation of MN (containing any whole chromosome) with the main nucleus, measured as the relative frequency of single MN synchronously condensed with the main nucleus in colchicine-treated prophase cells, among all single MN in colchicine-treated prophase cells at the first mitosis after misdistribution. It is noteworthy that in cells treated with spindle poisons 80–90% of induced MN originated from whole chromosomes (Surrallés *et al.*, 1995; Huber *et al.*, 1996; Sgura *et al.*, 1997).
- $nd_{(7-11)}$ is the mean probability of MND for chromosome 7 or 11 measured as the mean frequency of binucleate cells with a 3/1 hybridization signal distribution for either probed chromosome minus the estimated burden both of artefacts due to FISH staining and of the 'engulfment effect' due to cyt B treatment (Minissi *et al.*, 1999). The mean value between chromosomes 7 and 11 was multiplied by 23 in order to obtain an overall frequency of MND comparable to *cmls* and *ctdl*, under the assumption that all chromosomes behave similarly (Eastmond and Pinkel, 1990; Marshall *et al.*, 1996).

Table V. Comparisons between observed and expected frequencies (%) of induced trisomic M_2 cells in cultured human lymphocytes treated with colchicine

Colchicine concn	Trisomic M_2 cells		
	Observed frequency[a]	Expected frequency[b]	Observed/ expected
0	0.33	2.81[c]	0.12
10 nM	1.83	5.00[c]	0.37
25 nM	1.83	9.44[c]	0.19

Observed and expected frequencies were compared by the χ^2 test.
[a]Evaluated by chromosome counting in M_2 c-metaphases, FPG stained slides (see Table II).
[b]Calculated as follows: $f = syc(0.5cmsl + 0.25ctdl) + 23(0.5\ nd_{(7-11)}) + cnt$, where *syc* is the observed relative frequency of single MN synchronously condensed with the main nucleus in colchicine-treated M_2 prophases, *cmsl* and *ctdl* are the observed relative frequencies of ana-telophases with a single lagging chromosome or chromatid among all the M_1 ana-telophases, $nd_{(7-11)}$ is the mean frequency of binucleate cells with a 3/1 hybridization signal distribution for either probed chromosome minus the estimated artefact burden due to FISH staining (see Materials and methods) and the effect of cyt B (see Introduction) and *cnt* is the spontaneous frequency of trisomic M_1 c-metaphases in FPG stained slides.
[c]$P \ll 0.001$.

- *cnt* is the spontaneous frequency of trisomic cells for any chromosome measured as the frequency of trisomic M_1 c-metaphases in FPG stained slides.

Data obtained from cyt B-treated and untreated cultures were used in the same equation since it was demonstrated that, with the present protocol, the addition of 6 μg/ml cyt B does not interfere with the duration of the cell cycle (Minissi *et al.*, 1999).

The observed frequencies of M_2 trisomic metaphases are much lower than the expected ones, their ratios ranging between 1/6 and 1/3. Such a difference is highly significant using the χ^2 test (Table V). This suggests that the induction of a single chromosome misdistribution at anaphase does not result in a corresponding increase in the frequency of M_2 trisomic metaphases, at least within the time limit of our investigation. This remarkable difference might be due to a block of cell cycle progression which could result either in a mitotic delay or death of trisomic cells.

A similar evolution was observed by other authors: vinblastine-induced hyperdiploid bone marrow cells of mouse treated *in vivo* underwent a reduction in frequency in the cell population due to selection (Gustavino *et al.*, 1991); a comparison of induced aneuploidy in interphase nuclei with corresponding metaphase cells (Raimondi *et al.*, 1989) showed that hyperdiploidy was greater in interphase cells than in metaphases. The authors suggest that the observed discrepancy may represent a selective block against aneuploid cells. However, in a study on untreated human lymphocytes, similar incidences of hyperdiploid interphases and metaphases at the first mitosis *in vitro* were recorded (Carere *et al.*, 1998).

By FISH analysis it was possible to evaluate MND and consequently to compare the contribution of MND and MIND to the origin of trisomic karyotypes. Following the proposed equation, the contribution of MND is given by $23\times(0.5\ nd_{(7-11)}) = cMND$. The contribution of MIND is given by $syc(0.5\ cmsl + 0.25\ ctdl) = cMIND$. With the present data, for untreated cultures $cMND = 1.68\%$ and $cMIND = 0.07\%$ and their ratio is 24.0. For colchicine-treated cultures $cMND = 3.11\%$ and $cMIND = 0.83\%$ with 10 nM colchicine

and their ratio is 3.7. With 25 nM colchicine *cMND* = 6.94% and *cMIND* = 1.44% and their ratio is 4.8.

Following our data, in mitotically activated human lymphocytes treated *in vitro* with low concentrations of colchicine MND plays a more important role than MIND in the induction of trisomy.

A major role of MND in the origin of trisomic cells is also suggested by other authors, who observed that 3/1 binucleate cells are more frequent than FISH-positive MN in cultured human lymphocytes treated with spindle poisons (Marshall *et al.*, 1996; Elhajouji *et al.*, 1997; Sgura *et al.*, 1997). However, a more accurate comparison between MND and MIND was possible, allowing the finding of engulfment of MN in the main nucleus due to cyt B treatment (Minissi *et al.*, 1999).

In a previous paper, a major role of MIND compared with MND in the origin of trisomic cells was proposed on the basis of the relative frequencies of laggards, MN (in synchronous mitotic condensation with the main nucleus) and M_2 trisomic c-metaphases (Gustavino *et al.*, 1994). In the present paper the introduction of FISH analysis allowed us to quantify induced MND and to show its high incidence in colchicine-treated human lymphocytes. However, the observed frequency of induced M_2 trisomic c-metaphases does not correspond to the frequency of trisomic cells due to MND, suggesting the possibility of cell cycle lengthening or death among trisomic cells.

Even if our conclusions are restricted to data obtained on one single donor, yet they can be indicative of a more general process taking place in human lymphocytes.

Our data confirm the suitability of FISH on binucleate lymphocytes as a method for the analysis of chromosome segregation, even if a deeper understanding of the differences observed between approaches (FISH and chromosome counting) and of the evolutionary dynamics of cell populations is needed. As a conclusion, while in plant cells treated with low concentrations of a spindle poison MIND plays a major role in the induction of trisomy, in Chinese hamster cells MND prevails (Rizzoni *et al.*, 1989). In primary human lymphocytes MIND was demonstrated to take place (Gustavino *et al.*, 1994), however, the present study shows that its contribution is of minor importance, as compared with that of MND, in the production of trisomic karyotypes.

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