Mutagenesis vol.17 no.3 pp.233–239, 2002

Spontaneous and spindle poison-induced micronuclei and chromosome non-disjunction in cytokinesis-blocked lymphocytes from two age groups of women

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Fluorescence *in situ* hybridization (FISH) was used to evaluate spontaneous and aneuploidogen-induced micronucleus frequencies and non-disjunction of chromosomes X and 8 in cultured binucleated lymphocytes of women of two age groups. Demecolcine and vincristine were used as model aneuploidogens to induce micronuclei and chromosome malsegregation. Four of the women were aged 22–26 (mean 24.3) years and four 47–50 (mean 49.0) years. Pancentromeric FISH was applied to micronuclei to identify chromosomes and double-color centromeric FISH, performed in binucleates of two young and two older women, was used to assess the involvement of chromosomes X and 8 in micronuclei and non-disjunction. It was confirmed that age increases micronucleus frequency. Micronuclei containing whole chromosomes predominated in older females. Age also enhanced micronuclei containing acentric chromosome fragments. The inclusion of chromosomes X and 8 in micronuclei was enhanced by age and chromosome X was generally overrepresented. Non-disjunction of chromosomes X and 8 also increased with age, chromosome X being the more sensitive. Treatment of lymphocytes with vincristine and demecolcine increased micronucleus frequency and malsegregation of chromosomes X and 8 in both age groups. Comparison of the estimated frequencies of micronucleation and non-disjunction for all human chromosomes showed that non-disjunction is the main type of chromosome malsegregation.

Introduction

The cytokinesis block micronucleus assay first described by Fenech and Morley (1985) has been used by numerous researchers to investigate and assess chromosome damage in human lymphocytes. Anti-kinetochore antibodies (CREST) and fluorescence *in situ* hybridization (FISH), using centromeric probes, can be applied to discriminate micronuclei (MN) induced by chromosome breakage and missegregation of whole chromosomes (Kirsch-Volders *et al*., 1997). Using probes for specific chromosomes, non-disjunctional events can be assessed in cytokinesis-blocked interphase cells (Zijno *et al*., 1996a,b; Sgura *et al*., 1997).

Studies on the baseline frequency of MN in human lymphocytes have shown that the MN index is influenced by age and sex (Fenech and Morley, 1985, 1989). Similarly, Migliore *et al*. (1991) reported that age affects the basal frequency of micronucleated lymphocytes and is thus associated with an increased sensitivity to breakage or aneuploidogenic events. Additionally, Fenech *et al*. (1998) showed that MN frequency is significantly and positively correlated with age for males and females. Also, in a recent work based on 11 published studies (Peace and Succop, 1999) MN frequency was positively associated with age. In addition, after analysis of a large population sample, an increase in MN frequency was identified with age (Bolognesi *et al*., 1999). Recently, an analysis of results from 25 laboratories of nearly 7000 subjects confirmed the effect of age and gender on baseline MN frequency (Bonassi *et al*., 2001).

Jacobs *et al*. (1961) first described a correlation between aging and aneuploidy in cultured peripheral human lymphocytes, aneuploid cells being more prevalent in older persons. Subsequent studies confirmed this observation. Aneuploidy may have severe consequences for human health, including cancer and developmental abnormalities caused by altered gene dosage. Recently it was proposed that aneuploidy offers a mechanism of phenotype alteration which, above a certain threshold, is sufficient to cause all cancer-specific phenotypes and is independent of gene mutations (Duesberg *et al*., 1999; Li *et al*., 2000). Aneuploidy is also one of the main reasons for abortion (Hassold *et al*., 1996; Tucker and Preston, 1996). Chromosome lagging in anaphase and non-disjunction are the main mechanisms of chromosome malsegregation during mitosis, leading to aneuploid cells. Lagging of chromatids and chromosomes during anaphase results in micronucleus formation and has been considered a major cause of chromosome loss (Ford *et al*., 1988).

In aged females aneuploidy is mainly associated with chromosome X. Guttenbach *et al*. (1994) showed that the exclusion of sex chromosomes into MN doubles during a human life, from 11% in young probands to 20% in older ones. In addition, centromere-positive MN were significantly higher in older than in younger women (Catalán *et al*., 1995). Moreover, an overrepresentation of chromosome X was identified in lymphocyte micronuclei from older females as compared with younger ones (Catalán *et al*., 1995; Guttenbach *et al*., 1995), as well as in males (Hando *et al*., 1997; Catalán *et al*., 1998). Catalán *et al*. (2000b) also concluded that malsegregation of chromosome X is common in lymphocytes of both men and women and is more frequent than chromosome Y malsegregation.

The aim of the present study was to evaluate spontaneous and aneuploidogen-induced MN and non-disjunction rates in binucleate human lymphocytes and to compare how chromosome X and autosomes behave in relation to age and chemical treatment. Demecolcine and vincristine were used as model aneuploidogens. MN frequency and the contents of MN were studied using an alphoid pancentromeric probe in cytokinesis-blocked lymphocytes of two age groups of women, each consisting of four subjects. MN and non-disjunction of chromosomes X and 8 were also investigated by FISH analysis in four of the donors.

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Table I. Spontaneous and induced frequencies of micronucleated binucleated cells (BNMN) and micronuclei (MN) in lymphocyte cultures of eight female donors in two age groups

Donor	Age (years)	Untreated			Demecolcine treated (0.01 μg/ml)			Vincristine treated (0.03 μg/ml)		
		CBPI	‰BNMN	‰MN	CBPI	‰BNMN	‰MN	CBPI	‰BNMN	‰MN
1	26	1.93	7.31	7.85	1.91	13.57	14.12	1.83	19.94	22.15
2	26	1.77	8.39	9.53	1.92	14.39	15.73	1.85	17.52	21.90
3	23	1.84	7.14	7.57	1.73	14.93	15.40	1.71	17.71	24.59
4	22	1.80	7.72	7.88	1.86	15.55	15.93	1.72	17.32	20.55
Mean ± SE	24.25 ± 1.03	1.84 ± 0.04	7.64 ± 0.28	8.21 ± 0.45	1.86 ± 0.04	14.61 ± 0.42[a]	15.30 ± 0.41[a]	1.78 ± 0.04	18.12 ± 0.61[a]	22.30 ± 0.84[a]
5	47	1.86	24.72	29.20	1.94	29.72	34.24	1.74	62.50	79.81
6	50	1.72	17.50	19.45	1.69	25.85	26.93	1.64	39.69	50.34
7	50	1.84	25.50	28.30	1.86	28.85	31.67	1.75	60.96	75.71
8	49	1.76	21.55	26.17	1.74	23.98	29.24	1.74	48.66	62.98
Mean ± SE	49.00 ± 0.71	1.80 ± 0.03	22.32 ± 1.82[b]	25.78 ± 2.20[b]	1.81 ± 0.06	27.10 ± 1.33[b]	30.52 ± 1.57[b]	1.72 ± 0.03	52.95 ± 5.40[ab]	67.21 ± 6.67[ab]

The frequencies are shown per thousand binucleated cells. Lymphocyte proliferation is expressed by the CBPI.
[a]$P < 0.005$ in comparison with respective untreated cultures (Student's *t*-test).
[b]$P < 0.001$ in comparison with young female group (Student's *t*-test).

Materials and methods

Lymphocyte cultures and chemicals

Eight healthy adult women were included in this study. Four of them were 22–26 years old (mean age ± SE 24.25 ± 1.03 years) and four 47–50 years old (49.00 ± 0.71 years) (Table I). Whole blood (0.5 ml) derived from these women was added to 6.5 ml of Ham F-10 medium (Gibco), 1.5 ml of fetal calf serum (Gibco) and 0.2 ml of phytohemagglutinin (PHA) (Gibco). The culture medium was supplemented with the antibiotics penicillin/streptomycin (Gibco) and L-glutamine (Serva). Separate cultures were established from each donor corresponding to different experimental points. Cytochalasin-B (6 μg/ml) (Sigma) was added to the culture medium 44 h after culture initiation. Demecolcine and vincristine were added into the culture medium 41 h after culture initiation. The final concentration in the culture was 0.01 μg/ml for demecolcine (Sigma) and 0.03 μg/ml for vincristine sulfate (Sigma). The cultures were incubated at 37°C for 72 h in a 5% CO_2 atmosphere at 95% humidity.

Slide preparation

Cells were harvested 72 h after PHA stimulation. They were treated with a hypotonic solution of Ham F10:dH_2O (1:1) at 37°C for 2 min. Fixation of the cells was performed instantly at least three times, treating them with a solution of methanol:acetic acid (3:1) at room temperature. Cell drops were layered onto clean slides and stored at 4°C for *in situ* hybridization. For estimation of micronucleus frequency, at least 1000 binucleated cells were scored for each donor and for each treatment.

FISH analysis

Pancentromeric probe

The procedure followed has been published elsewhere (Andrianopoulos *et al*., 2000). Briefly, the slides were pretreated with pepsin (Sigma) solution in 0.01 M HCl, pH 3, for 5 min, dehydrated with an increasing series of ethanol (Merck) and denatured in 70% formamide (Merck) for 2 min. The slides were covered with digoxigenin-conjugated α-satellite probe for all human centromeres (P5095:DG5; Oncor) and hybridized overnight at 37°C in a humidified chamber. At the end of the hybridization time the slides were washed in 50% formamide in 2× SSC (saline sodium citrate buffer). Immunodetection of the probe was achieved with anti-digoxigenin antibody (D-8156; Sigma), anti-mouse antibody (P-8547; Sigma) and fluoroscein isothiocyanate (FITC)-conjugated anti-sheep antibody (F-7634; Sigma). Counterstaining was performed with a mixture of 4′,6-diamidino-2-phenylindole (DAPI; Sigma) and propidium iodide (P.I; Sigma) and slides were mounted in Vectashield mounting medium (Vector Laboratories).

Specific chromosome X and 8 probes

The slides were treated with 25 μg/ml pepsin, dehydrated and denatured as described above. Hybridization was performed at 37°C overnight with a mixture of chromosome X α-satellite digoxigenin-conjugated probe DXZ1 (CP5060-DG.5; Oncor) and chromosome 8 α-satellite biotin-conjugated probe D8Z2 (CP5013-B.5; Oncor). Repeated washings were performed with 50% formamide at 37°C for 10 min. Immunodetection of the chromosome X probe was achieved with anti-digoxigenin antibody (D-8156; Sigma), anti-mouse IgG antibody (P-8547; Sigma) and FITC-conjugated anti-sheep IgG antibody (F-7634; Sigma). Immunodetection of the chromosome 8 probe was achieved with rhodamine 600–avidin D antibody (A2005; Vector) and biotin-conjugated monoclonal anti-avidin antibody (B-9655; Sigma). Counterstaining was performed with DAPI and the slides were mounted in Vectashield mounting medium (Vector Laboratories).

Slide scoring

The slides were kept in the dark at 4°C. Slides hybridized with the pancentromeric probe were analyzed under a Zeiss Axioskop epifluorescence microscope. The bandpass filters used were of 546, 490 and 360 nm for green, blue and UV light, respectively. At least 50 MN were analyzed for the presence of a centromere-positive signal for each experimental point. A centromere-positive micronucleus was recorded when the signal was of the same intensity as that of the main nucleus. MN were characterized only in those cells whose nuclei contained clear pancentromeric signals. For the scoring of MN standard criteria were used (Fenech, 1993). To determine possible cytotoxic effects, 2000 cells were counted for calculation of the cytokinesis block proliferation index (CBPI) (Surrallés *et al*., 1995), which is given by the formula $CBPI = M_1 + 2M_2 + 3(M_3 + M_4)/n$, where M_1, M_2, M_3 and M_4 correspond to the numbers of cells with one, two, three and four nuclei and n is the total number of cells. Slides simultaneously hybridized with chromosome X and 8 probes were analyzed under a Zeiss Axioskop epifluorescence microscope equipped with a triple bandpass filter for the simultaneous detection of rhodamine, FITC and DAPI. Cells with malsegregation were also observed under the 546 and 490 nm bandpass filters. The distribution patterns of the hybridization signals were considered as follows: binucleated cell with normal signal distribution for chromosome X or 8, 2:2; single non-disjunction for chromosome X or 8, 3:1; double non-disjunction for chromosome X or 8, 4:0; double non-disjunction including both chromosomes in the same cell, 3:1/3:1. Two signals very close to or touching each other or connected with a thin hybridized bridge were recorded as one and only two signals clearly separated from each other were recorded as two (Eastmond *et al*., 1995). Frequencies were expressed as the numbers of binucleated lymphocytes with an abnormal signal distribution per thousand binucleated cells analyzed. At least 1000 binucleated cells were counted for each experimental point to detect non-disjunction. Only binucleated cells with the correct signal number, four spots for each chromosome, irrespective of their distribution, were scored. Micronucleation and non-disjunction were scored independently for chromosomes X and 8, but recorded in parallel per cell.

Statistical analysis

Data on MN induction, determined by pancentromeric probe FISH analysis, were statistically analyzed by Student's *t*-test comparing mean frequencies. Statistical analysis between various experimental points of malsegregated cells hybridized for chromosomes X and 8 was performed using the G-test (Sokal and Rohlf, 1981) for independence on 2×2 tables. This test is based on the general assumption of χ^2 analysis, but offers theoretical and computational advantages. Mean frequencies for total micronucleation (%BNC^+MN) and total non-disjunction (TND) at various experimental points were statistically analyzed by one-way analysis of variance (ANOVA). %BNC^+MN is an estimate of micronucleation as a whole among the four subjects studied for chromosome X and 8 malsegregation and was expressed as binucleated micronucleated cells containing pancentromeric hybridization signal as a percentage of the total binucleated cells analyzed in the experiments with the pancentromeric probe. TND is an estimation of non-disjunction for the total genome for the same subjects and was calculated by multiplying the initial

Table II. Spontaneous and induced frequencies of micronuclei (MN) with (C^+MN) and without (C^-MN) a centromeric signal in binucleated (BN) lymphocytes treated *in vitro* with 0.01 μg/ml demecolcine and 0.03 μg/ml vincristine, as evaluated after FISH analysis using an α-satellite probe for all human centromeres in eight female donors representing two age groups

Donor	Untreated				Demecolcine treated (0.01 μg/ml)				Vincristine treated (0.03 μg/ml)			
	BN cells scored	MN	C^+MN (‰)	C^-MN (‰)	BN cells scored	MN	C^+MN (‰)	C^-MN (‰)	BN cells scored	MN	C^+MN (‰)	C^-MN (‰)
Young												
1	7520	59	26 (3.46)	33 (4.39)	7150	100	48 (6.71)	52 (7.27)	2257	50	41 (18.17)	9 (3.99)
2	5244	50	23 (4.39)	27 (5.15)	5977	93	33 (5.52)	60 (10.04)	2283	50	30 (13.14)	20 (8.76)
3	9109	69	29 (3.18)	40 (4.39)	2143	33	20 (9.33)	13 (6.07)	2033	50	35 (17.22)	15 (7.38)
4	6344	50	17 (2.68)	33 (5.20)	2637	42	20 (7.58)	22 (8.34)	2482	51	36 (14.50)	15 (6.04)
Mean ± SE			3.43 ± 0.36	4.78 ± 0.23			7.29 ± 0.80[a]	7.93 ± 0.84[a]			15.76 ± 1.17[a]	6.54 ± 1.02
Old												
5	5582	163	118 (21.14)	45 (8.06)	1548	53	40 (25.84)	13 (8.40)	1040	83	64 (61.54)	19 (18.27)
6	2571	50	31 (12.06)	19 (7.39)	1857	50	33 (17.77)	17 (9.15)	1033	52	48 (46.47)	4 (3.87)
7	3569	101	74 (20.73)	27 (7.57)	1768	50	35 (19.80)	15 (8.48)	1017	77	60 (59.00)	17 (16.72)
8	1949	51	34 (17.44)	17 (8.72)	1710	50	38 (22.22)	17 (7.02)	1048	66	52 (49.62)	14 (13.36)
Mean ± SE			17.84 ± 2.10[b]	7.94 ± 0.30[b]			21.41 ± 1.74[b]	8.26 ± 0.45			54.16 ± 3.62[ab]	13.06 ± 3.23

Frequencies are expressed per thousand binucleated cells.
[a]$P \leq 0.01$ in comparison with respective untreated cultures (Student's *t*-test).
[b]$P \leq 0.0005$ in comparison with young female group (Student's *t*-test).

frequency of non-disjunction for chromosome 8 by 22 and adding the non-disjunction frequency of chromosome X.

Results

A comparison between the two age groups in relation to CBPI, BNMN and MN frequency is presented in Table I. Regarding CBPI, in the untreated cultures no statistical difference was observed between the two groups. Statistically significantly higher BNMN and MN frequencies were seen in the older group (22.32 ± 1.82 and 25.78 ± 2.20) than the younger one (7.64 ± 0.28 and 8.21 ± 0.45). Demecolcine treatment had no impact on CBPI and increased MN frequencies significantly only in the young group (14.61 ± 0.42). No statistically significant differences were observed in the older group. Considering the two groups, the BNMN and MN frequencies after demecolcine treatment were still significantly higher in the older than the younger persons. After vincristine treatment no statistically significant differences were observed in CBPI. BNMN (18.12 ± 0.61 for the young and 52.95 ± 5.40 for the older group) and MN (22.30 ± 0.84 for the young and 67.21 ± 6.67 for the older group) frequencies were statistically significantly higher in relation to untreated groups. The older women also exhibited significantly higher frequencies in comparison with the younger ones after vincristine treatment.

The content of micronuclei was determined by FISH analysis, using an α-satellite pancentromeric probe. As can be seen in Table II, the older women exhibited higher C^-MN and C^+MN (7.94 ± 0.30 and 17.84 ± 2.10) frequencies compared with the younger women (4.78 ± 0.23 and 3.43 ± 0.36), which indicates a higher rate of chromosome breakage and whole chromosome micronucleation with increasing age. In the older women C^+MN frequencies were more than twice C^-MN frequencies. A significant increase in both C^+MN (7.29 ± 0.80) and C^-MN (7.93 ± 0.84) was shown after treatment of cells from the young subjects with demecolcine. No increase was observed in the older subjects. Vincristine presence in the culture enhanced C^+MN frequencies in both age groups (15.76 ± 1.17 and 54.16 ± 3.62). On the other hand, no increase in C^-MN frequencies induced by vincristine was seen in either group. The distribution of the numbers of centromeric signals (data not shown) showed that on average 82% (young subjects) and 62% (old subjects) of spontaneous MN contained only one pancentromeric signal. The proportion of such MN was also higher in the young than in the older subjects in cultures treated with demecolcine and vincristine.

Chromosome X and 8 malsegregation was studied in two of the four donors from each group, nos 3 and 4 from the younger group (mean age ± SE 22.50 ± 0.50 years) and nos 5 and 8 from the older group (48.00 ± 1.00 years). The results on chromosome X and 8 micronucleation are presented in Table III. Frequencies of MN, estimated per thousand binucleated lymphocytes, containing a hybridization signal for chromosome X (MNX^+) or 8 ($MN8^+$) with one signal (MNX^{1+} and $MN8^{1+}$) and two or more signals ($MNX^{\geq 2+}$) are shown. In the untreated cultures of the young women only chromosome X was included in MN and the frequency of MN with one chromosome X (2.14 ± 0.47) was prevalent in relation to MN with two or more chromosomes X (0.48 ± 0.19). In the older group the frequencies of MNX^+ (15.64 ± 4.37), MNX^{1+} (9.51 ± 1.53) and $MNX^{\geq 2+}$ (6.13 ± 2.84) were significantly higher than in the younger group. MN containing chromosome 8 were rarer (1.05 ± 0.36). Addition of demecolcine to the cultures from the young females resulted in increased frequencies of MNX^+ (4.75 ± 0.25), MNX^{1+} (3.12 ± 0.12), $MNX^{\geq 2+}$ (1.63 ± 0.13) and $MN8^+$ (0.55 ± 0.05). Vincristine treatment of lymphocyte cultures from the young women statistically significantly enhanced every type of micronuclei, MNX^+ (10.52 ± 0.52), $MN8^+$ (2.04 ± 0.04), MNX^{1+} (7.64 ± 0.64) and $MNX^{\geq 2+}$ (2.88 ± 0.12), in comparison with untreated cultures. In vincristine-treated cultures from the older females significantly higher frequencies of MNX^+ (32.00 ± 4.00), $MN8^+$ (8.50 ± 2.50), MNX^{1+} (21.50 ± 1.50) and $MN8^{1+}$ (7.50 ± 2.50) were observed in comparison with the untreated cultures from the older females. Comparing MN frequencies in both untreated and chemically treated cultures, significantly higher MNX^+, MNX^{1+} and $MNX^{\geq 2+}$ frequencies were observed in the older women than in the young ones. This observation was also true for $MN8^+$ and $MN8^{1+}$ frequencies, but only for vincristine-treated cultures. Frequencies of MN harboring both chromosomes in the same binucleated lymphocyte were very low

Table III. Spontaneous and induced micronucleus (MN‰) frequencies in four female donors representing young (donors 3 and 4) and older (donors 5 and 8) age groups, for chromosomes X and 8 after FISH with chromosome-specific centromeric probes

Culture	Donor	BN cells scored	MN	Chromosome X			Chromosome 8	
				MNX^{+} (‰)	MNX^{1+} (‰)	$MNX^{\geq 2+}$ (‰)	$MN8^{+}$ (‰)	$MN8^{1+}$ (‰)
Untreated	3	3450	26	10 (2.90)	9 (2.61)	1 (0.29)	0 (0)	0 (0)
	4	3000	22	7 (2.33)	5 (1.67)	2 (0.67)	0 (0)	0 (0)
	Mean ± SE			2.62 ± 0.29	2.14 ± 0.47	0.48 ± 0.19	0	0
	5	1450	50	29 (20)	16 (11.03)	13 (8.96)	1 (0.69)	1 (0.69)
	8	2130	50	24 (11.27)	17 (7.98)	7 (3.29)	3 (1.41)	3 (1.41)
	Mean ± SE			15.64 ± 4.37[a]	9.51 ± 1.53[a]	6.13 ± 2.84[a]	1.05 ± 0.36[a]	1.05 ± 0.36[a]
Demecolcine treated	3	3400	42	17 (5)	11 (3.23)	6 (1.76)	2 (0.59)	1 (0.29)
(0.01 µg/ml)	4	2000	28	9 (4.5)	6 (3)	3 (1.50)	1 (0.50)	1 (0.50)
	Mean ± SE			4.75 ± 0.25[b]	3.12 ± 0.12	1.63 ± 0.13	0.55 ± 0.05	0.40 ± 0.11
	5	1305	50	30 (22.99)	16 (12.26)	14 (10.73)	0 (0)	0 (0)
	8	1680	50	26 (15.48)	16 (9.52)	10 (5.95)	2 (1.19)	2 (1.19)
	Mean ± SE			19.24 ± 3.76[a]	10.89 ± 1.37[a]	8.34 ± 2.39[a]	0.60 ± 0.60	0.60 ± 0.60
Vincristine treated	3	1450	41	16 (11.03)	12 (8.28)	4 (2.76)	3 (2.07)	3 (2.07)
(0.03 µg/ml)	4	1000	28	10 (10)	7 (7)	3 (3)	2 (2)	2 (2)
	Mean ± SE			10.52 ± 0.52[c]	7.64 ± 0.64[c]	2.88 ± 0.12[c]	2.04 ± 0.04[c]	2.04 ± 0.04[c]
	5	1000	82	36 (36)	23 (23)	13 (13)	11 (11)	10 (10)
	8	1000	68	28 (28)	20 (20)	8 (8)	6 (6)	5 (5)
	Mean ± SE			32.00 ± 4.00[ac]	21.50 ± 1.50[ac]	10.50 ± 2.50[a]	8.50 ± 2.50[ac]	7.50 ± 2.50[ac]

BN, binucleated cells; MNX^{+} and $MN8^{+}$, micronuclei containing hybridization signal for chromosome X and 8, respectively; MNX^{1+} and $MNX^{\geq 2+}$, micronuclei containing one and two or more hybridization signals for chromosome X; $MN8^{1+}$, micronuclei containing one hybridization signal for chromosome 8. One micronucleus with more than one chromosome 8 signal was observed for demecolcine treatment of donor 3 and vincristine treatment of donors 5 and 8.

[a]$P < 0.05$ in comparison with young female group (G-test).

[b]Borderline effect in comparison with respective untreated cultures (G-test).

[c]$P < 0.01$ in comparison with respective untreated cultures (G-test).

and were seen only in chemically treated cultures from the older females.

The results of the chromosome non-disjunction study are presented in Table IV. In untreated cells from young females only single events of type 3:1 were observed, while no double events were seen. The frequency of non-disjunction was statistically higher in untreated cultures from the older persons for both chromosomes (1.45 ± 0.25 for chromosome X and 0.50 ± 0.20 for chromosome 8), and a low frequency of double events was seen. In demecolcine-treated cultures from the young females non-disjunction frequencies for chromosomes X (2.25 ± 0.15) and 8 (0.60 ± 0.10) were significantly different from the corresponding frequencies of the untreated cultures (0.65 ± 0.05 and 0.05 ± 0.05). In the older females non-disjunction for chromosome X (4.15 ± 0.35) was statistically significantly higher in demecolcine-treated than in untreated cultures. Enhanced non-disjunction frequencies for both chromosomes studied were also observed in cells from the older women in relation to the young ones. Double events of the distribution type 3:1/3:1 were seen in demecolcine-treated cultures from both groups studied. Addition of vincristine to the cultures significantly increased frequencies of chromosome X (3.70 ± 0.30 and 7.50 ± 0.50) and chromosome 8 (1.95 ± 0.15 and 3.65 ± 0.25) non-disjunction in both the young and older women; the frequencies were significantly higher for the older than for the younger females. Cells which exhibited non-disjunction with the distribution 3:1/3:1 were present in both groups but were more common in the older women.

Chromosome non-disjunction frequencies for the total genome (TND) were also calculated by multiplying the initial frequency of non-disjunction for chromosome 8 by 22 and adding the non-disjunction frequency for chromosome X. It was assumed that non-disjunction occurs randomly and independently for each autosome (Kirsch-Volders *et al*., 1996). TND was found to be enhanced in the older females in comparison with the young ones in untreated cultures (12.45 ± 4.15 and 1.75 ± 1.05). Addition of either demecolcine or vincristine to the cultures increased TND in both age groups in relation to untreated cultures. In addition, TND was also significantly higher in the older than the younger subjects in demecolcine-treated (27.25 ± 0.75 and 15.45 ± 2.35) and vincristine-treated (87.80 ± 6.00 and 46.60 ± 3.00) cultures. An estimation of chromosome micronucleation as a whole among the four subjects studied, based on $BNC^{+}MN$ frequencies, was also made. $\%BNC^{+}MN$ was expressed as binucleated micronucleated cells which contained a pancentromeric hybridization signal as a percentage of the total binucleated cells analyzed in the experiments with the pancentromeric probe. When the participation of $\%BNC^{+}MN$ and TND in total chromosome malsegregation was compared, TND was observed to be higher than $\%BNC^{+}MN$ in every treated culture in relation to untreated cultures for both age groups. However, chromosome X micronucleation and non-disjunction frequencies in untreated cultures from the older women were shown to be the same (Tables III and IV).

Frequencies of MN containing hybridization signals for chromosome X as part of the total MN containing a pancentromeric hybridization signal for donors 3, 4, 5 and 8 were calculated by multiplication of the respective $C^{+}MN$ frequencies (Table II) by the percentage of MNX^{+} (Table III) (Richard *et al*., 1994). Frequencies of MN containing autosomes, including chromosome 8, were estimated by subtracting the calculated MNX^{+} frequencies from the total $C^{+}MN$ frequencies (Catalán *et al*., 1995, 1998; Surallés, *et al*., 1996a). The results are presented in Figure 1. In comparison with the younger women, MN frequency in the older women was ~10-fold for chromosome X and ~5-fold for autosomes. The respective increases were ~4-fold and 2-fold in demecolcine-treated cultures and ~4-fold and 3-fold in vincristine-treated cultures. These results

Table IV. Spontaneous and induced non-disjunction frequencies (%ND) in four female donors representing young (donors 3 and 4) and older (donors 5 and 8) age groups for chromosomes X and 8 after FISH with chromosome-specific centromeric probes

Culture	Donor	BN cells scored	Chromosome X				Chromosome 8				Both X/8 3:1/3:1	TND	%BNC$^+$ MN
			2:2	3:1	4:0	%ND	2:2	3:1	4:0	%ND			
Untreated	3	1000	993	7	0	0.7	1000	0	0	0	0	0.7	0.29
	4	1000	994	6	0	0.6	999	1	0	0.1	0	2.8	0.27
	Mean ± SE					0.65 ± 0.05				0.05 ± 0.05		1.75 ± 1.05	0.28 ± 0.01
	5	1000	983	16	1	1.7	997	3	0	0.3	0	8.3	1.83
	8	1000	988	9	2	1.2	993	5	1	0.7	1	16.6	1.49
	Mean ± SE					1.45 ± 0.25[a]				0.50 ± 0.20[a]		12.45 ± 4.15	1.66 ± 0.17[b]
Demecolcine treated	3	1000	976	18	4	2.4	993	5	0	0.7	2	17.8	0.89
(0.01 µg/ml)	4	1000	979	14	4	2.1	995	2	0	0.5	3	13.1	0.72
	Mean ± SE					2.25 ± 0.15[c]				0.60 ± 0.10[c]		15.45 ± 2.35[d]	0.81 ± 0.09[d]
	5	1000	955	33	8	4.5	990	5	1	1.0	4	26.5	2.20
	8	1000	962	25	7	3.8	989	3	2	1.1	6	28	1.81
	Mean ± SE					4.15 ± 0.35[ac]				1.05 ± 0.05		27.25 ± 0.75[b]	2.01 ± 0.20[b]
Vincristine treated	3	1000	960	30	4	4	982	8	4	1.8	6	43.6	1.38
(0.03 µg/ml)	4	1000	966	22	5	3.4	979	9	5	2.1	7	49.6	1.29
	Mean ± SE					3.70 ± 0.30[c]				1.95 ± 0.15[c]		46.6 ± 3.0[d]	1.34 ± 0.05[d]
	5	1000	920	59	11	8	961	21	8	3.9	10	93.8	5
	8	1000	930	48	10	7	966	11	11	3.4	12	81.8	3.91
	Mean ± SE					7.50 ± 0.50[ac]				3.65 ± 0.25[ac]		87.8 ± 6.0[bd]	4.46 ± 0.55[bd]

Frequencies are estimated as binucleated cells with the indicated distribution of hybridization signals as a percentage of the total binucleated cells analyzed. Total chromosome non-disjunction (TND) frequencies are estimated by adding the chromosome X non-disjunction frequency to 22× the chromosome 8 non-disjunction frequency (assuming that non-disjunction occurs randomly and independently for autosomes). Frequencies of total micronucleation (%BNC$^+$MN) are expressed as binucleated (BN) micronucleated cells containing a centromeric signal (C$^+$MN) as a percentage of total binucleated cells analyzed in the experiments with the pancentromeric probe.
[a]$P < 0.05$ in comparison with young female group (G-test).
[b]$P < 0.05$ in comparison with young female group (one-way ANOVA).
[c]$P \leqslant 0.005$ in comparison with respective untreated cultures (G-test).
[d]$P \leqslant 0.05$ in comparison with respective untreated cultures (one-way ANOVA).

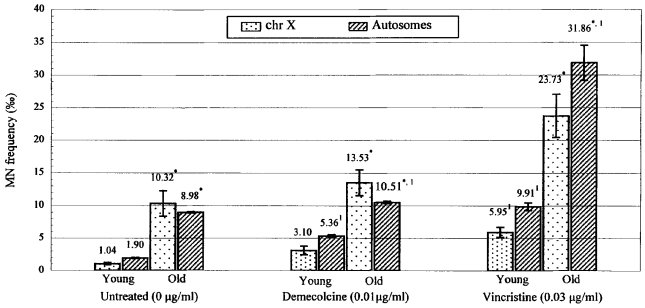

Fig. 1. Comparison of the involvement of chromosome X and autosomes in total micronucleation, spontaneously or as induced by demecolcine and vincristine, in two young women aged 22 and 23 years and two older women aged 47 and 49 years. Frequencies were calculated by multiplying centromere-positive MN frequencies (C$^+$MN) of the same donors (from experiments with a pancentromeric probe) by the percentage of MN containing hybridization signals for chromosome X (MNX$^+$). Frequencies of MN-containing autosomes, including chromosome 8, were estimated by subtracting the calculated MNX$^+$ frequencies from the total C$^+$MN frequencies. chrX and Autosomes, mean MN frequencies for chromosome X and autosomes, respectively. The error bars represent the SEM. *$P < 0.05$ in comparison with young female group (one-way ANOVA). [1]$P < 0.05$ in comparison with respective untreated cultures (one-way ANOVA).

suggest that age has a stronger effect on micronucleation of chromosome X than that of autosomes.

Discussion

We found that micronucleus frequency is higher in both treated and untreated lymphocyte cultures of older than younger women. This indicates an age effect, which is in accordance with several earlier studies. Bolognesi *et al*. (1999) also supported an increase in basal level of micronuclei with age. Fenech (1998) found that MN frequency is significantly and positively correlated with age for both genders with a regression line of 0.314 in males and 0.517 in females. Barale *et al*. (1998) observed that age and sex are by far the most important variables associated with modifications in MN frequency.

By FISH analysis, using an α-satellite pancentromeric probe, we have shown that MN frequency in women increases with age due to both chromosome breakage and whole chromosome missegregation. An age-related increase in baseline frequencies of chromosome aberrations in humans has recently been reviewed (Bolognesi *et al*., 1997). Cultures treated with demecolcine showed increased C$^+$MN frequencies, but in young women C$^-$MN frequencies were also increased. Demecolcine (colcemid), a synthetic derivative of colchicine, was also found to induce chromosome breakage by Matsuoka *et al*. (1993), who reported a slight increase in structural aberrations when CHO cells were treated with 0.05 and 1.0 µg/ml colcemid or vincristine. In addition, Arni and Hertner (1997) observed that colcemid and colchicine, at high concentrations, induced numerous structural chromosomal aberrations in diploid and tetraploid CHO cells, while vincristine induced chromosome breakage only in tetraploid cells. In the present study treatment of female lymphocytes with vincristine resulted in increased C$^+$MN frequencies, but C$^-$MN frequencies did not show any significant increase in either age group.

Since the first report by Jacobs *et al.* (1961) of enhanced chromosome loss with age, a lot of papers supporting this finding have appeared. This has been shown by chromosome analysis in metaphases and FISH analysis in interphase cells (Nowinski *et al*., 1990; Hando *et al*., 1994; Surrallés *et al*., 1996a,b).

Specific probes for chromosome X and one autosome, chromosome 8, were used in our study. Our results confirm

and expand observations of several recent publications. Richard *et al*. (1994) found that X aneuploidy and the frequency of chromosome X per MN increased with age. According to Catalán *et al*. (1995), the presence of centromere-positive MN was significantly higher among older than younger females, with a high overrepresentation of chromosome X in the MN. Zijno *et al*. (1996b) reported that age increased malsegregation of chromosomes X, 8 and 18 in lymphocytes of male donors. Catalán *et al*. (1998) showed that chromosome X was also preferentially micronucleated in male lymphocytes, although X micronucleation was much more common in women than in men. Recent results suggested that high micronucleation and loss of chromosome X in female lymphocytes is due to distal lagging behind in anaphase (Catalán *et al*., 2000a). In addition, Carere *et al*. (1999) showed that aging is positively correlated with the incidence of malsegregation in male subjects and confirmed the higher susceptibility to malsegregation of chromosome X in comparison with autosomes. Chromosome X sensitivity to aneuploidy was suggested to be due to premature centromere division and modified centromere function (Fitzgerald, 1983; Nakagome *et al*., 1984). Abbruzo *et al*. (1985) presented results from 45,X and 47,XXX females, suggesting that the inactive chromosome X was the missing or extra one. Tucker *et al*. (1996) analyzed the MN content of two females with a different X;9 reciprocal translocation and concluded that the inactive chromosome X is preferentially included in the MN. Hando *et al*. (1997) determined a 10-fold difference in the frequency of chromosome X-positive MN in 46,XX females compared with 46,XY males and 45,X females, providing further support that the chromosome X in MN is the inactive chromosome. However, other investigators failed to demonstrate preferential malsegregation of the inactive X homolog (Surrallés *et al*., 1996b; Zijno *et al*., 1996a; Catalán *et al*., 2000a). A comparison of the involvement of chromosome X and autosomes showed that age affects chromosome X micronucleation at a higher rate in comparison with autosomes as a whole. However, the possibility that the frequency of autosome loss is higher than estimated in this study cannot be excluded, due to the lethality of monosomic lymphocytes.

Age, demecolcine and vincristine increased both total micronucleation and TND frequencies. Comparison of the estimated frequencies of total micronucleation (%BNC$^+$MN) and total non-disjunction (TND) showed that non-disjunction is the main mechanism producing aneuploidy, both spontaneous and chemically induced, in younger as well as older females. It can be argued that TND is just an estimate, while %BNC$^+$MN is based on real data. If %BNC$^+$MN is similarly estimated to TND, then the differences between micronucleation and non-disjunction would be smaller, but non-disjunction is still the prevalent mechanism producing chromosome malsegregation. Similarly, it has been reported that the contribution of chromosome non-disjunction to total aneuploidy was determined to be more important than micronucleation, irrespective of the inducing agent (Kirsch-Volders *et al*., 1996; Elhajouji *et al*., 1997; Sgura *et al*., 1997; Touil *et al*., 2000). However, Carere *et al*. (1999) concluded that in males chromosome X displays similar rates of non-disjunction and micronucleation at variance with autosomes, which showed negligible rates of micronucleation compared with non-disjunction.

Our results can be summarized as follows.

In females:

- age increases MN frequency;
- MN containing whole chromosomes predominate in older females and age also enhances MN containing acentric chromosome fragments;
- the inclusion of chromosome X and autosomes in spontaneous and aneuploidogen-induced MN is enhanced by age, with a higher representation of chromosome X;
- spontaneous and spindle poison-induced non-disjunction of chromosome X and autosomes increases with age, chromosome X being the more sensitive;
- the involvement of non-disjunction, estimated as TND, is prevalent in relation to chromosome micronucleation, %BNC$^+$MN, in spontaneous and spindle poison-induced total chromosome malsegregation in young as well as older females.

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Received on May 1, 2001; accepted on January 14, 2002