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Age-Dependent Inclusion of Sex Chromosomes in Lymphocyte Micronuclei of Man

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Summary

Two-color centromeric FISH was used to study the inclusion of the X and Y chromosomes in micronuclei of cultured lymphocytes from 10 men representing two age groups (21–29 years and 51–55 years). In addition, pancentromeric FISH was separately performed to identify any human chromosomes in micronuclei. One hundred micronuclei per probe were examined from each donor. A higher mean frequency of Y-positive micronuclei was observed in the older men than in the younger men. In both age groups, the X chromosome was micronucleated clearly more often than expected by chance, and the Y chromosome was overrepresented in micronuclei among the older men but not among the younger men. In lymphocytes of four women, X-positive micronuclei were more frequent than they were in men, even after the fact that women have two X chromosomes was taken into account. Similar results were obtained in first-division lymphocytes identified by cytochalasin-B–induced cytokinesis block. In comparison with normal cells, these binucleate cells showed a higher frequency (per 1,000 nuclei) of X-positive micronuclei (in the older men) but a lower frequency of micronuclei harboring autosomes or acentric fragments. In conclusion, the results show that both the X chromosome and the Y chromosome are preferentially micronucleated in male lymphocytes, the Y chromosome only in older subjects. Although the X chromosome has a general tendency to be included in micronuclei, it is micronucleated much more often in women than in men, which is probably the main reason for the high micronucleus frequency in women that has been documented in many previous studies.

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Introduction

Jacobs et al. (1961) were the first to describe an association between chronological aging and aneuploidy in metaphase spreads of cultured human lymphocytes, and subsequent studies showed that this phenomenon mainly concerns the loss of the X chromosome in women and the loss of the Y chromosome in men (Fitzgerald and McEwan 1977; Galloway and Buckton 1978; Richard et al. 1993; Guttenbach et al. 1995; Stone and Sandberg 1995). In addition to nondisjunction, chromosome lagging behind in anaphase, with subsequent elimination by micronucleation at the time of nuclear-membrane formation, has been proposed as a mechanism accounting for the aneuploidy (Ford et al. 1988).

The involvement of micronucleation in age-related chromosome loss has been supported by several studies showing that the rate of micronucleus (MN) formation increases with age, especially in women (Högstedt et al. 1983; Fenech and Morley 1986; Ganguly 1993; Fenech et al. 1994). The use of either antikinetochore antibodies (Fenech and Morley 1989; Hando et al. 1994) or FISH with a pancentromeric DNA probe (Catalán et al. 1995; Scarpato et al. 1996) has revealed that the age-dependent increase of MNs in women's lymphocytes is due to MNs harboring whole chromosomes instead of acentric fragments. Although some studies also have indicated the involvement of autosomes (Richard et al. 1994; Catalán et al. 1995), this phenomenon has largely been explained as involving the X chromosome. FISH studies using X chromosome–specific centromeric DNA probes showed a high overrepresentation of the X chromosome in lymphocyte MNs of women, with a strong tendency toward rate increases with age (Guttenbach et al. 1994; Hando et al. 1994; Richard et al. 1994; Catalán et al. 1995). The exceptional role of the X chromosome in both chromosome loss and MN formation has been suggested as being due to the inactive X chromosome (Fitzgerald 1975; Nakagome et al. 1983; Abruzzo et al. 1985; Tucker et al. 1996), although a recent study did not demonstrate preferential micronucleation of the inactive X (Surrallés et al. 1996*b*).

X chromosome missegregation was also found to in-

Table 1

Frequency of MNs and No. of C+ MNs in Human Lymphocytes Cultured With (Binucleate Cells) and Without (Mononucleate Cells) Cytochalasin B

		MNs in			
		Mononucleate cells		Binucleate cells	
Subject	Age (Years)	Total MNs[a]	C+ MNs[b]	Total MNs[a]	C+ MNs[b]
Men:					
1	54	14.5	41	10.2	53
2	55	20.5	42	14.5	54
3	52	15.5	51	13.0	64
4	51	7.0	44	8.5	36
5	51	7.0	40	6.7	42
Mean	52.6	12.9	43.6	10.6	49.8
6	29	10.5	53	6.2	46
7	21	16.5	38	5.7	38
8	29	7.0	39	8.2	37
9	24	8.0	38	3.0	40
10	24	13.0	43	6.2	48
Mean	25.4	11.0	42.2	5.9	41.8
Women:					
11	58	13.0	50	11.5	59
12	59	34.5	33	11.5	53
13	29	13.0	46	7.5	62
14	26	29.5	41	15.5	50
Mean	43.0	22.5	42.5	11.5	56.0

[a] Frequency (per 1,000 cells), scored from 2,000 cells/sample.
[b] Scored from 100 MNs/sample.

crease with age in men (Zijno et al. 1996), and a recent study has suggested that the (active) X chromosome in males and in Turner syndrome in females is also preferentially micronucleated (Hando et al. 1997). The inclusion of the Y chromosome in MNs, as well as Y-chromosome loss, has been shown to be clearly age dependent (Guttenbach et al. 1994, 1995; Nath et al. 1995).

At the present time, the frequency of MNs is usually evaluated on the basis of cytokinesis-blocked binucleate cells produced by cytochalasin B (Cyt-B), since this approach allows the identification of cells after their first in vitro division (Fenech and Morley 1986). Recent studies have suggested, however, that there are dissimilarities, in the contents of MNs, between binucleate cells and cells cultured without Cyt-B, dissimilarities that may reflect either differences between first-division cells and cultured cells in general or consequences of the cytokinesis block (Norppa et al. 1993; Surrallés et al. 1996*a;* Falck et al. 1997).

In the present study, an X chromosome–specific centromeric DNA probe was used to examine whether the age-dependent increase in X chromosome–containing MNs, earlier observed in women, could also be shown in the lymphocytes of men. Since the Y chromosome was simultaneously detected, it was possible to assess, for the first time, the micronucleation of both the Y chromosome and the (active) X chromosome in the same cells. Furthermore, a pancentromeric DNA probe was applied to study the inclusion of any human chromosomes in lymphocyte MNs; the probe also allowed us to estimate the frequencies of MNs harboring autosomes and acentric fragments. Lymphocytes from women were inspected, for comparison. All analyses were performed with and without Cyt-B, to gain insight into the possible influence that cytokinesis block has on the contents of MNs.

Material and Methods

Cell Cultures and Slide Preparation

Heparinized blood samples were obtained from a total of 14 healthy nonsmokers—10 men and 4 women—distributed equally between two age groups: <30 years and >50 years (see table 1). Four separate samplings were performed, always involving both age groups. Informed consent was obtained from each donor.

Mononuclear leukocytes were isolated, and phytohemagglutinin-stimulated lymphocyte cultures were established at an initial cell density of 1.5×10^6/ml, as described in detail elsewhere (Norppa et al. 1993). For each donor, cultures with and without Cyt-B (Sigma) were included. Cyt-B at a final concentration of 6 μg/ml was added 24 h after the cultures were started. After 65 h incubation at 37°C, microscopic slides were prepared by use of a cytocentrifuge (6 min at 400 rpm; Shandon Cytospin 2). The slides were air-dried, fixed for 20 min in cold (−20°C) methanol, and stored at −20°C.

FISH

The slides were pretreated with a mild pepsin solution (5 μg/ml in 0.01 N HCl, pH 3) and were washed briefly in distilled water and PBS. They were then postfixed in 1% (v/v) formaldehyde, washed with PBS, and dehydrated in an increasing series of ethanol. The DNA of the cells was denatured in 70% formamide/2 × SSC at 70° C for 2 min and was dehydrated.

For the identification of the centromeres of any chromosome, a digoxigenin-labeled DNA probe containing centromeric alphoid sequences from all human chromosomes (P5095; Oncor) was prewarmed at 37°C for 5 min, denatured for 5 min at 70°C, and chilled on ice. Each slide received 5 μl of the probe and then was covered with a glass coverslip, sealed with rubber cement, and incubated overnight at 37°C in a moist chamber. The slides were then washed at 37°C, twice with 50% formamide/2 × SSC (for 10 min each time), twice in 2 × SSC (for 4 min each time), and once in 4 × SSC/0.05% Tween-20 (for 5 min), followed by incubation in

4 × SSC/0.5% skimmed milk (for 15 min at 37°C). After a short wash in 4 × SSC/ 0.05% Tween-20, the slides were incubated (at 37°C in a moist chamber) with antidigoxigenin antibody (1:250; Sigma), FITC-conjugated anti-mouse antibody (1:20; Boehringer-Mannheim), and FITC-conjugated anti-sheep antibody (1:20; Sigma). Each incubation lasted 30 min and was followed by three washes in 4 × SSC/ 0.05% Tween-20 (for 2 min each at 37°C). After the last wash, the slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide and were mounted in antifade solution, as described elsewhere (Catalán et al. 1995).

For the detection of the X and Y chromosomes, the prehybridization steps as well as the hybridization were performed as described above, except that hybridization mixtures were prepared for the probes. For the detection of the X chromosome in women, the hybridization mixture contained 64% (v/v) formamide, 5% dextran sulfate, 2 × SSC, 700 μg herring-sperm DNA/ml, and 0.95 μg biotin-labeled chromosome-X alpha-satellite probe/ml (DXZ1; Oncor). For the simultaneous detection of the X and Y chromosomes in men, the hybridization mixture contained 62% formamide, 5% dextran sulfate, 2 × SSC, 0.95 μg biotin-labeled chromosome-X alpha-satellite probe/ml (DXZ1; Oncor), and 0.95 μg digoxigenin-labeled chromosome-Y alpha-satellite probe/ml (DYZ3; Oncor). After overnight hybridization, the slides were washed twice in 50% formamide/2 × SSC (for 10 min each time, at 43°C), three times in 0.1 × SSC (for 4 min each time, at 37°C), and once in 4 × SSC/0.05% Tween-20 (for 5 min at 37°C), followed by incubation (for 15 min at 37°C) in 4 × SSC/0.5% skim milk and a short wash in 4 × SSC/0.05% Tween-20. The X chromosome was detected by alternating layers of fluorescein-conjugated Avidin D (1:100; Vector), biotinylated antiavidin D (1:100; Vector), and fluorescein-conjugated Avidin D (1:100). For the detection of both the X and Y chromosomes, the aforementioned incubations also included, respectively, mouse anti-digoxigenin antibody (1:250), rabbit anti-mouse-tetramethylrhodamine-isothiocyanate (TRITC) antibody (1:50; Sigma), and anti-rabbit-TRITC antibody (1:50; Sigma). These incubations were performed at 37°C, for 30 min each, with three washes in 4 × SSC/0.05% Tween-20 (for 2 min each time, at 37°C) between the incubations. Finally, the slides were stained with a 4 × SSC/0.05% Tween-20 solution containing DAPI (10 μg/ml) for 5 min in the dark and were washed in tap water, air-dried, and mounted in antifade solution.

Microscopy

The slides were coded and were scored by two microscopists under a Leitz Laborlux S microscope equipped with epifluorescence and filter blocks A, I3, and N2. A total of 100 MNs (50 MNs by each scorer) per culture and probe were separately analyzed for the presence of all human centromeres and either the X chromosome (in women) or the X and Y chromosomes simultaneously (in men). MNs, identified by DAPI fluorescence (filter block A), were checked for the presence of FITC (all centromeres; X chromosome) and TRITC signals (Y chromosome), by use of filter blocks I3 and N2, respectively. Altogether, 5,600 MNs were inspected for the presence of sex-chromosome or pancentromeric signals.

For FISH involving either the X chromosome or the X and Y chromosomes simultaneously, 200 nuclei were scored to evaluate hybridization efficiency. MN contents were scored only when >90% of the main nuclei showed two signals. Inspection of metaphases has shown that the "all-human-centromeres probe" labels every centromere, including that of the Y chromosome. Since the actual efficiency that this probe (or any other pancentromeric probe) has in the identification of centromeres in MNs is not known, MNs were characterized only in cells in which the FITC signals were clearly visible and homogeneously dispersed within the main nuclei. Although centromere-positive (C^+) MNs might, in principle, harbor either products of structural chromosome aberrations (fragments with centromeric sequences, and exchanges that have had problems in cell division) or fragments in addition to whole chromosomes, such rare situations are not considered to contribute markedly to baseline MNs. Therefore, C^+ MNs were defined as being those harboring whole chromosomes and centromere-negative MN acentric fragments.

Statistical Evaluation

To compare the means of the two age groups in men, a repeated-measures analysis of variance in which age was the independent variable was performed by use of the GLM procedure of the SAS software package (SAS Institute 1989, pp. 891–996). Culture type and its interaction with age were added to the model. To compare men and women, sex and various interactions between sex and other variables were included; the frequencies of X-positive (X^+) MNs in women were divided by 2, to take into account the fact that women have two X chromosomes. To compare mononucleate (i.e., without Cyt-B) and binucleate (i.e., with Cyt-B) cells accurately, the MN frequencies of the binucleate cells were divided by 2. Thus, all MN frequencies were expressed per 1,000 nuclei. Before performing the analysis of variance for the men, we tested assumptions of normality by use of the Shapiro-Wilk test (Shapiro and Wilk 1965; Royston 1982) and tested equality of variances by use of a folded *F*-test (Steel and Torrie 1980). For X^+ MNs and Y^+ MNs, the hypothesis for either normality or variance

homogeneity had to be rejected, and the analyses-of-variance models were calculated by use of ranks instead of the original values. All models including sex were analyzed by use of ranks.

A *t*-test was used to test the hypothesis that the mean prevalence of sex chromosomes among whole chromosome–containing MNs coincides with the expected random involvement of a chromosome in MNs with one, two, or three chromosomes (2.22%). This was done separately for the young and the old men and for mononucleate and binucleate cells. In addition, to check whether one of the sex chromosomes was included in MNs more often than the other, the prevalences of X^+ MNs and Y^+ MNs among C^+ MNs were compared by use of a *t*-test for paired observations. All *t*-tests were two sided.

Results

Figure 1 shows examples of micronucleated binucleate lymphocytes hybridized with centromeric DNA probes specific for the X chromosome and for the X and Y chromosomes simultaneously; the cell in figure 1*d* harbors two MNs—one X^+ and the other Y^+. On average, almost half of all MNs in the men (42.2% in the young without Cyt-B and 43.6% in the old without Cyt-B; 41.8% and 49.8%, respectively, in the same groups with Cyt-B) were labeled positively with the pancentromeric DNA probe and thus contained alphoid DNA sequences common to all human chromosomes (table 1). Such MNs are expected to harbor whole chromosomes, and unlabeled MNs should represent acentric fragments. More than half of the C^+ MNs contained single centromeric signals, approximately one fourth contained two signals, and 8.8%–18.3% of the MNs contained three or more signals (data not shown).

A Y^+ signal was observed in ~10% of all MNs from the older men, with or without Cyt-B (table 2)—that is, 4.0–6.5 times more often than in the young men. Also X^+ MNs (table 3) were more common in the older men (7.0% without Cyt-B and 15.2% with Cyt-B) than in the younger men (4.8% and 6.2%, respectively). The majority of X^+ MNs and Y^+ MNs had only one signal, indicating the presence either of a single chromatid or of sister chromatids with overlapping centromeric labels (tables 2 and 3); the rest almost exclusively contained two X signals or two Y signals, probably representing both sister chromatids.

The frequencies of different types of MNs were calculated from the data of tables 1, 2, and 3 and are presented in table 4. These figures were used as a basis for the analysis of variance in the effects of sex, age, and culture type (table 5).

The contribution of autosomes was estimated by subtracting the Y^+ and X^+ MNs from the total of C^+ MNs. Autosomes constituted approximately one-fourth of all

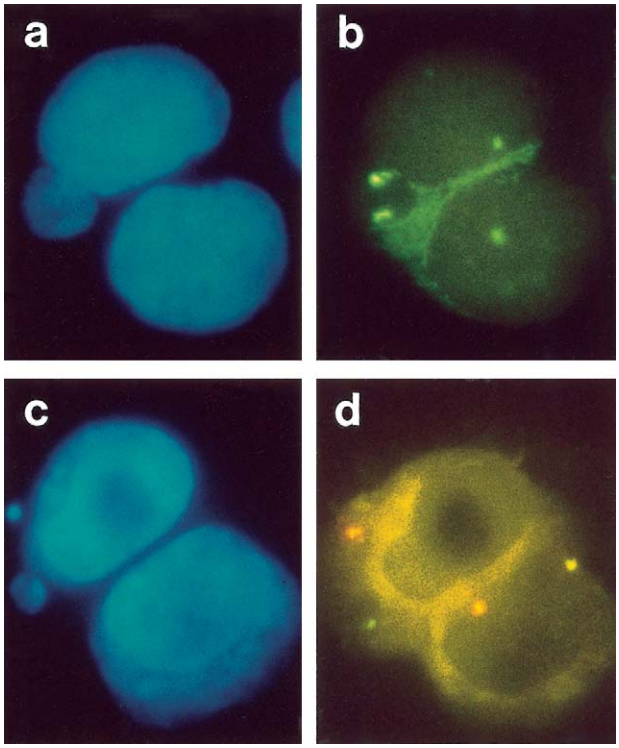

Figure 1 Micronucleated binucleate lymphocytes hybridized in situ with centromeric alpha-satellite DNA probes. The cells are shown under UV excitation (*a* and *c*), blue excitation (*b*), or combined, blue-and-green excitation (*d*). *a* and *b*, Cell of a woman, showing two X-chromosome signals in an MN, hybridized with biotinylated DXZ1 detected by FITC. *c* and *d*, Cell of a man, harboring two MNs—one X^+, hybridized with biotinylated DXZ1 detected by FITC (*green*), and the other Y^+, hybridized with digoxigenin-labeled DYZ3 detected by TRITC (*red*).

MNs in the older men (26.2% without Cyt-B and 25.0% with Cyt-B) and approximately one-third of those in the younger men (35.8% and 33.2%, respectively). As shown in tables 4 and 5, this difference between the two age groups was explained by low frequencies of other types of MNs in the young men. The frequency of autosome-containing MNs was not significantly affected by age.

The mean frequency of Y^+ MNs was clearly higher in the older men (1.6/1,000 cells without Cyt-B and 1.1/1,000 cells with Cyt-B; tables 4 and 5) than in the younger men (0.2/1,000 and 0.1/1,000, respectively). An age effect was also observed in the frequency of X^+ MNs, although age alone did not have a statistically significant influence (tables 4 and 5). The analysis of variance showed, however, a significant interaction between age and culture type, reflecting the facts that the effect of age on X^+ MNs was clearer in binucleate cells than in mononucleate cells and that the frequency of X^+ MNs in the older men was higher in binucleate cells than in

Table 2

No. of Y^+ MNs in Male Lymphocytes Cultured With (Binucleate Cells) and Without (Mononucleate Cells) Cytochalasin B

	No. of MNs with Signals[a]							
	Mononucleate Cells				Binucleate Cells			
Age (Years)	Total	With 1 Signal	With 2 Signals	With ⩾3 Signals	Total	With 1 Signal	With 2 Signals	With ⩾3 Signals
54	11	9	1	1	13	8	5	0
55	17	8	8	1	11	10	1	0
52	16	12	4	0	16	12	4	0
51	3	2	1	0	4	2	2	0
51	5	5	0	0	4	4	0	0
Mean	10.4	7.2	2.8	.4	9.6	7.2	2.4	.0
29	3	3	0	0	6	6	0	0
21	1	1	0	0	1	1	0	0
29	1	1	0	0	1	1	0	0
24	0	0	0	0	3	2	1	0
24	3	3	0	0	1	1	0	0
Mean	1.6	1.6	.0	.0	2.4	2.2	.2	.0

[a] In 100 MNs scored/person.

mononucleate cells. The older men also had higher mean frequencies of fragment-containing MNs (table 4) and of total MNs (table 1), particularly in binucleate cells, but for these classes of MNs the age effect was not statistically significant.

If it is assumed that all chromosomes have the same probability of being included in MNs, any single chromosome should appear in 1 (2.17%) of 46 MNs harboring a whole chromosome. If MNs with two and three chromosomes are taken into account , the expected figure can be estimated to be 2.22% ($100 \times [1/46 + 1/46 \times 1/45 + 1/46 \times 1/45 \times 1/44]$). If it is assumed that all X^+ MNs and Y^+ MNs are also C^+, the prevalence of the sex chromosomes among whole-chromosome–

Table 3

No. of X^+ MNs in Human Lymphocytes Cultured With (Binucleate Cells) and Without (Mononucleate Cells) Cytochalasin B

	No. of MNs with Signals[a]							
	Mononucleate Cells				Binucleate Cells			
Age (Years)	Total	With 1 Signal	With 2 Signals	With ⩾3 Signals	Total	With 1 Signal	With 2 Signals	With ⩾3 Signals
Men:								
54	1	1	0	0	14	9	5	0
55	7	4	3	0	20	16	4	0
52	19	15	4	0	30	12	18	0
51	4	4	0	0	7	7	0	0
51	4	4	0	0	5	4	1	0
Mean	7.0	5.6	1.4	.0	15.2	9.6	5.6	.0
29	3	3	0	0	3	1	2	0
21	5	5	0	0	4	4	0	0
29	2	2	0	0	3	3	0	0
24	7	7	0	0	4	2	2	0
24	7	5	1	1	17	9	8	0
Mean	4.8	4.4	.2	.2	6.2	3.8	2.4	.0
Women:								
58	30	19	10	1	50	29	18	3
59	25	14	11	0	38	21	17	0
29	13	8	4	1	33	18	13	2
26	36	13	19	4	46	34	10	2
Mean	26.0	13.5	11.0	1.5	41.8	25.5	14.5	1.8

[a] In 100 MNs scored/person.

containing MNs can easily be calculated. In the older men, the Y chromosome was included in C^+ MNs clearly more often (23.6% in those without Cyt-B [$P = .03$] and 18.1% in those with Cyt-B [$P = .008$]; 2-sided t-test) than would be expected by chance; in the younger men (who had values of 3.6% and 5.6%, respectively), the rate did not significantly deviate from the theoretical value. The average contribution of the X chromosome among C^+ MNs of the male donors was, in all cases, much higher than the theoretical value; the difference was statistically significant for all men combined (13.4% without Cyt-B [$P = .006$] and 21.2% with Cyt-B [$P = .002$]), for the mononucleate cells of the young men (11.7% [$P = .025$]), and for the binucleate cells of the older men (28.3% [$P = .014$]). In comparison with Y^+ MNs, the contribution of X^+ MNs among C^+ MNs was higher in the mononucleate cells of the younger men and in the binucleate cells of the older men, but the differences were not statistically significant ($P = .06$).

Although the present study was focused on men, four women were also included, to allow comparison between sexes. Significantly higher values were obtained in the women than in the men, for the frequency of X^+ MNs (tables 4 and 5). The X chromosome constituted, on the average, as much as 68.8% (in those without Cyt-B) and 78.6% (in those with Cyt-B) of C^+ MNs in the older women and 56.3% and 67.9%, respectively, in the younger women. In both men and women, the total frequency of MNs/1,000 nuclei was significantly higher in mononucleate cells of ordinary cultures than it was in binucleate cells, in cultures containing Cyt-B (tables 1 and 5); this was due to higher frequencies of MNs harboring fragments and autosomes (tables 4 and 5).

Discussion

The main aim of the present study was to characterize the contents of MNs in men, with a special emphasis on the sex chromosomes and aging. As far as we know, no previous studies are available on the simultaneous identification of the X and the Y chromosomes in MNs.

The analysis of the Y chromosome in a total of 2,000 MNs (with and without Cyt-B) showed that Y^+ MNs are a major contributor to the age-dependent increase of MNs in men and that the Y chromosome is highly overrepresented in MNs of older men. These results are in accordance with earlier findings by Guttenbach et al. (1994) and Nath et al. (1995), who used smaller numbers of scored MNs. In our study, the average proportion of Y^+ MNs among all MNs was lower (2.4% in the younger men and 9.6% in the older men) than those observed by Guttenbach et al. (1994), for children (14%) and old men (20%), or by Nath et al. (1995), for male donors of different ages (13.5%). Previously, an age-dependent increase has also been observed in the frequencies of interphase nuclei lacking a Y-chromosome signal, in cultured male lymphocytes (Guttenbach et al. 1995). The excess of both Y-chromosome micronucleation and loss in lymphocytes seems to affect only men >40–50 years of age, which agrees with previous findings on Y-chromosome loss in bone marrow (see Stone and Sandberg 1995). However, the results of Nath et al. (1995) suggest that a considerable portion of older men do not show an elevated rate of Y-chromosome micronucleation. In the present study, subjects 4 and 5, who also had low frequencies of X^+ MNs, appear to belong to this category. The reason for this variation is unknown.

We also found an age effect for the frequency of X^+ MNs, especially in binucleate cells produced by Cyt-B. This finding is in accordance with the recent results of Hando et al. (1997), who saw an age-dependent increase in the frequency of X^+ MNs in binucleate lymphocytes of 18 male newborns, 35 adult men (22–79 years of age), and 7 Turner syndrome females. Our observations also agree with those of Zijno et al. (1996), who reported an age-dependent increase in the frequency of malsegregation of the X chromosome in binucleate lymphocytes of men, although these authors did not show data for MNs and nondisjunction separately. Under the assumption that every chromosome has the same probability of being micronucleated, the X chromosome was highly overrepresented in the MNs of both age groups of men in the present study, in agreement with the results that Hando et al. (1997) found in males and Turner syndrome females. In the young men, the X chromosome was more commonly found in MNs than was the Y chromosome. The overrepresentation of the X chromosome could be seen both with and without Cyt-B and cannot, therefore, be explained by an unspecific effect of cytokinesis block. It is presently unknown whether the X chromosome is preferentially micronucleated also in male meiotic cells; this is an interesting question, since the loss of the X chromosome in Turner syndrome appears to be mainly of paternal origin (Jacobs et al. 1997).

Many studies have shown that the frequency of MNs is higher in women than in men (Narod et al. 1988; Fenech et al. 1994; Bonassi et al. 1995; Nath et al. 1995; Scarpato et al. 1996). A possible explanation for this difference is the preferential micronucleation of the X chromosome, which has been shown to occur in lymphocytes of women, both in vitro and in vivo (Guttenbach et al., 1994; Hando et al. 1994; Richard et al. 1994; Catalán et al. 1995; Surrallés et al. 1996*a*), and which is not, therefore, a culture artifact. The present study showed that the sex difference in the frequency of MNs is mainly due to X^+ MNs. In agreement with the recent findings by Hando et al. (1997), the micronucleation of the X chromosome was much higher in women than in men and could not be explained only by the fact that

Table 4

Frequency of MNs Harboring Acentric Fragments, Autosomes, the X Chromosome, or the Y Chromosome, in Human Lymphocytes Cultured With (Binucleate Cells) or Without (Mononucleate Cells) Cytochalasin B

Age (Years)	Frequency (per 1,000 Nuclei) of MNs Harboring							
	Fragments		Autosomes		X Chromosome		Y Chromosome	
	Mononucleate	Binucleate	Mononucleate	Binucleate	Mononucleate	Binucleate	Mononucleate	Binucleate
Men:								
54	8.6	4.8	4.2	2.7	.1	1.4	1.6	1.3
55	11.9	6.7	3.7	3.3	1.4	2.9	3.5	1.6
52	7.6	4.7	2.5	2.3	2.9	3.9	2.5	2.1
51	3.9	5.4	2.6	2.1	.3	.6	.2	.3
51	4.2	3.9	2.2	2.2	.3	.3	.4	.3
Mean (SD)	7.2 (3.3)	5.1 (1.1)	3.0 (.9)	2.5 (.5)	1.0 (1.2)	1.8 (1.5)	1.6 (1.4)	1.1 (.8)
29	4.9	3.4	4.9	2.3	.3	.2	.3	.4
21	10.2	3.6	5.3	1.8	.8	.2	.2	.1
29	4.3	5.2	2.5	2.7	.1	.2	.1	.1
24	5.0	1.8	2.5	1.0	.6	.1	.0	.1
24	7.4	3.3	4.3	1.9	.9	1.1	.4	.1
Mean (SD)	6.4 (2.5)	3.5 (1.2)	3.9 (1.3)	1.9 (.6)	.6 (.3)	.4 (.4)	.2 (.2)	.1 (.1)
Women:								
58	6.5	4.7	2.6	1.0	3.9	5.7		
59	23.1	5.4	2.8	1.7	8.6	4.4		
29	7.0	2.8	4.3	2.2	1.7	2.5		
26	17.4	7.7	1.5	.6	10.6	7.1		
Mean (SD)	13.5 (8.1)	5.2 (2.0)	2.8 (1.2)	1.4 (.7)	6.2 (4.1)	4.9 (2.0)		

women have two X chromosomes. This finding may reflect the preferential micronucleation of the inactive X.

If it is assumed that the active X is micronucleated at a similar rate in men and women, our results from men would predict a contribution of 64%–92% for the inactive X among X^+ MNs in women. This coincides both with the findings by Abruzzo et al. (1985), who reported an 85% contribution of the late-replicating X chromosome in X-aneuploid female lymphocytes, and with those by Tucker et al. (1996), who reported an 83.3% proportion of untranslocated (inactive) X chromosome among X^+ MNs in lymphocytes of women who had the balanced X;9 reciprocal translocation. A recent study in women (Surrallés et al. 1996*b*), in which the inactive X was identified by the lack of histone H4 acetylation, suggested, however, that there are no differences, in MN formation, between the inactive and active X chromosomes. Surrallés et al. (1996*b*) concluded that the high micronucleation of the X chromosome is explained by a constitutive defect in the machinery involved in the mitotic segregation of the X chromosome.

Centromere dysfunction due to either faulty kinetochore proteins or premature centromere division (PCD) has been suggested as the mechanism by which the (inactive) X and, possibly, also the Y chromosome form MNs and are lost in aging subjects (Fitzgerald 1975; Fitzgerald and McEwan 1977; Nath et al. 1995). This view is supported by observations showing that 56.3% ("active" X) and 70.9% ("inactive" X) (Tucker et al. 1996) and 36.9% (Hando et al. 1994) of X^+ MNs in women and 87.8% of Y^+ MNs in men (Nath et al. 1995) are kinetochore negative. In 45,X females and normal males, kinetochore was lacking in 68.2% of X^+ MNs (Hando et al. 1997). On the other hand, Stone and Sandberg (1995) reported that they have not seen significant rates of PCD of the Y chromosome in >5,000 bone-marrow samples, although Y-chromosome loss is common in the bone marrow of old men. In addition to centromere defects, the age-dependent shortening of telomeres, resulting in instability of the chromosome ends,

Table 5

***P* Values for Influence of Sex, Age, and Culture Type on Frequency of Different Categories of MNs in the Multivariate Analysis of Variance**

Endpoint	*P* for		
	Men		
	Age	Culture Type	Sex[a]
Total no. of MNs	.17	.01	.07
X^+ MNs[a]	.12[b]	.78	.02[c]
Y^+ MNs[a]	.01	.43	NA
MNs with autosomes	.75	.007	.17
MNs with fragments	.28	.02	.15

[a] The model was calculated by the use of ranks. NA = not applicable.

[b] A significant age × culture-type interaction ($P = .048$) was observed.

[c] In women, the frequencies of X^+ MNs were divided by 2, to take into account that women have two X chromosomes.

either in the short arm (Guttenbach et al. 1995) or in the long arm (Stone and Sandberg 1995), may be important in the process of Y-chromosome loss. Our finding that the X chromosome is common in MNs of men irrespective of age and that the Y chromosome is preferentially micronucleated only with aging suggests that the mechanisms involved are different for the two sex chromosomes.

The present results further support our earlier findings (Surrallés et al. 1996*a;* Falck et al. 1997) indicating that the frequencies of X^+ MNs (in older subjects) are higher, but that those of MNs containing autosomes and acentric fragments are lower, in binucleate lymphocytes compared with conventionally cultured lymphocytes. Differences in the composition of the cell population might explain the findings. Binucleate cells are thought to constitute a rather uniform population of cells after their first in vitro division, whereas many cells in ordinary 65–72-h cultures have probably divided more than once, although some have not divided at all. On the other hand, the results may reflect the abnormal nature of a cytokinesis-blocked binucleate cell. In such a cell, lagging acentrics and autosomes might preferentially be included within the main nuclei or be extruded, or MN expression may be delayed (Surrallés et al. 1996*a;* Falck et al. 1997). Whatever the reason, the X and Y chromosomes are affected differently, since the frequencies of Y^+ MNs were not higher in binucleate cells than they were in mononucleate cells.

In summary, the present study shows that both the X and the Y chromosomes are overrepresented in lymphocyte MNs of men but that the Y chromosome is overrepresented only in older subjects. Although the X chromosome is preferentially micronucleated, even in young subjects, in both men and women, women show a much higher rate of X^+ MNs than would be predicted by the fact that they have two X chromosomes. The difference, in MN frequency, between the sexes, which has been documented in many previous studies, appears to be due primarily to MNs harboring the X chromosome.

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