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Sex Chromosome Loss and Aging: In Situ Hybridization Studies on Human Interphase Nuclei

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Summary

A total of 1,000 lymphocyte interphase nuclei per proband from 90 females and 138 males age 1 wk to 93 years were analyzed by in situ hybridization for loss of the X and Y chromosomes, respectively. Both sex chromosomes showed an age-dependent loss. In males, Y hypoploidy was very low up to age 15 years (0.05%) but continuously increased to a frequency of 1.34% in men age 76–80 years. In females, the baseline level for X chromosome loss is much higher than that seen for the Y chromosome in males. Even prepubertal females show a rate of X chromosome loss, on the order of 1.5%–2.5%, rising to ~4.5%–5% in women older than 75 years. Dividing the female probands into three biological age groups on the basis of sex hormone function (<13 years, 13–51 years, and >51 years), a significant correlation of X chromosome loss versus age could clearly be demonstrated in women beyond age 51 years. Females age 51–91 years showed monosomy X at a rate from 3.2% to 5.1%. In contrast to sex chromosomal loss, the frequency of autosomal monosomies does not change during the course of aging: Chromosome 1 and chromosome 17 monosomic cells were found with a constant incidence of 1.2% and 1%, respectively. These data also indicate that autosome loss in interphase nuclei is not a function of chromosome size.

Introduction

In the early 1960s, Jacobs et al. (1961, 1963) published the first reports on increased aneuploidy in human lymphocytes with advancing age. Since that time numerous investigations have confirmed that hypoploidy increases with age and is more common than hyperploidy in metaphase spreads. Employing banding techniques, the chromosomes lost most frequently were identified as the X chromosome in females and the Y chromosome in males (Neurath et al. 1970; Fitzgerald and McEwan 1977; Galloway and Buckton 1978). The age-associated loss of the sex chromosomes was not only analyzed in healthy probands, but also in patients with histories of reproductive problems (Horsman et al. 1987; Nowinski et al. 1990), in cancer patients (UKCCG 1992; Hunter et al. 1993; Riske et al. 1994), in patients with Alzheimer disease (Kormann-Bortolotto et al. 1993), and in patients with Down syndrome (Percy et al. 1993).

Most of these investigations have in common that they are based on metaphase analyses. Although the metaphase method permits the simultaneous assessment of numerical and structural aberrations (Ganguly 1993), the technique is time consuming and prone to technical artifacts such as chromosome loss during slide preparation. This problem can be overcome by application of the in situ hybridization technique with chromosome-specific DNA probes, allowing the direct analysis of large numbers of interphase nuclei.

In the present investigation, the technique of in situ hybridization with chromosome-specific DNA probes was applied in order to determine the frequency of Y and X chromosome loss in male and female probands of different ages. DNA probes for chromosome 1 and chromosome 17 were selected as autosomal controls.

Probands, Material, and Methods

Probands

Lymphocyte nuclei from 90 healthy female and 138 male probands age 1 wk to 93 years were analyzed for X and Y chromosome loss, respectively. To allow for optimal age discrimination, probands were divided into age groups of 5-year intervals, each group represented by at least four males and at least four females.

Lymphocyte nuclei of a subset of the probands (one proband per age group) were investigated for loss of chromosome 1 and chromosome 17.

Blood Cultures

Lymphocyte cultures from the probands were set up in RPMI 1640 medium supplemented with 15% FCS. Cell proliferation was stimulated with phytohemagglutinin. The cells were harvested after 72 h culture time.

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Hypotonic treatment of the cells was performed in 0.075 M KCl for 7 min at 37°C. The cells were fixed in methanol:glacial acetic acid (3:1) overnight, and, after washing in fixative, were dropped onto clean slides. Preparations were stored at −20°C.

DNA Probes

The probes *pXBR* (Yang et al. 1982; Willard et al. 1983), *pHY2.1* (Cooke 1976), and *pUC1.77* (Cooke and Hindley 1979) were used as X-, Y-, and chromosome 1–specific DNA probes, respectively. The probes were labeled with biotin-16-dUTP by nick translation. A biotinylated probe specific for chromosome 17 was purchased from Oncor.

In Situ Hybridization

Slides were denatured in 70% formamide/2 × SSC at 70°C for 20 s to 2 min and then dehydrated by passing through a chilled alcohol series. Twenty microliters of hybridization mixture (50% formamide, 2 × SSC, 5% dextran sulphate, and 10 ng biotinylated probe) were denatured for 5 min at 70°C, chilled on ice, and then applied to each slide. Hybridization was performed overnight at 37°C. Depending on the probe used, the slides were washed three times at 40°C (*pXBR*, *pUC1.77*, chromosome 17–specific probe) or at room temperature (*pHY2.1*) in 50% formamide/2 × SSC, followed by two washes in 1 × SSC and 0.1 × SSC.

Probe detection with streptavidine-horseradish-peroxidase and diaminobenzidine (DAB) was carried out as described elsewhere (Schmid et al. 1990). FISH was done as described by Dominguez-Steglich et al. (1992).

Scoring of Cells

A total of 1,000 hybridized interphase nuclei per donor were analyzed. Screening of the preparations was done without knowledge of the age of the proband. Only slides showing efficient hybridization were evaluated.

The Y chromosomal DNA probe *pHY2.1* hybridizes to highly repetitive sequences in the heterochromatic region of the long arm of the human Y chromosome. Furthermore, it detects small sequences in the telomeric regions of some autosomes and the X chromosome (Cooke et al. 1982). These hybridization signals are minor, but they could be used in the present study as markers for the accessibility of the chromatin for the probe. Nuclei lacking the strong Y chromosomal signal, but exhibiting faint signals involving other chromosomes,

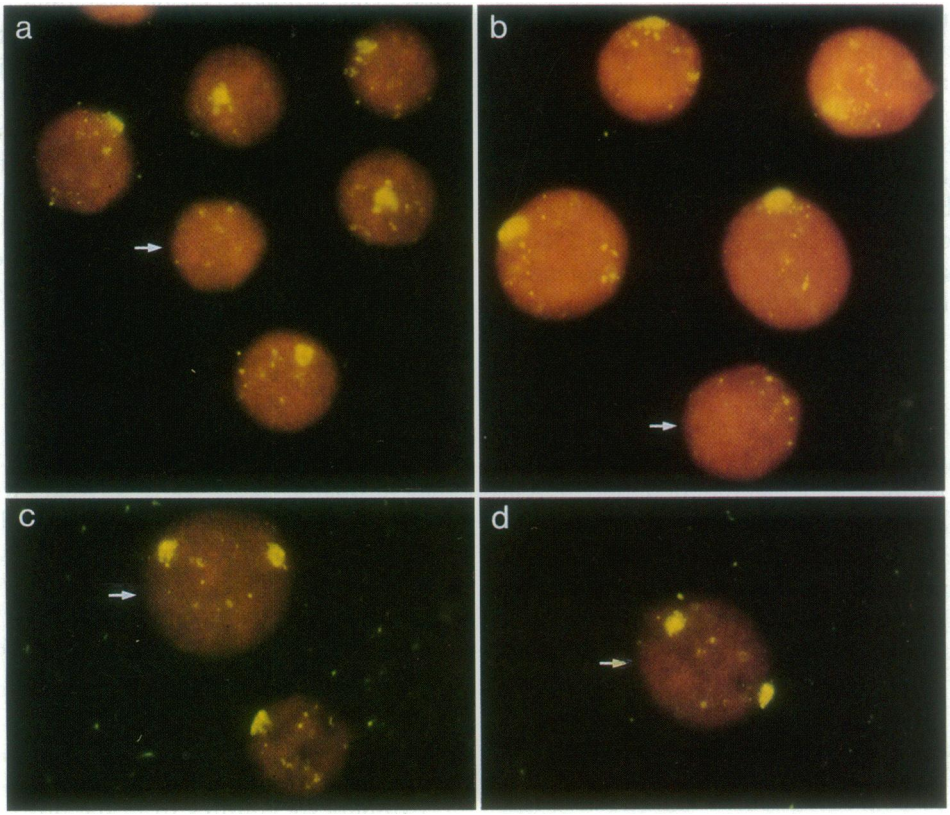

Figure 1 Interphase nuclei of a male proband hybridized in situ with biotinylated *pHY2.1* DNA. Signals were detected by avidin-FITC. *a* and *b*, Nuclei lacking a hybridization signal are indicated by arrows. *c* and *d*, Nuclei exhibiting two hybridization signals are indicated by arrows.

Table 1

Incidence (%) of X and Y Hypoploidy in Directly Isolated Lymphocytes and Lymphocytes Cultured for 48 h and 72 h, of Female and Male Donors

	Directly Isolated Lymphocytes		48 h Cultures		72 h Cultures	
Age	X loss	Y loss	X loss	Y loss	X loss	Y loss
1 wk				.0		.0
18 years		.2	2.5		2.3	.2
24 years	1.3	.3	1.5	.2	1.5	.2
33 years	3.8	.3			3.5	.4
52 years		.5	3.8		4.0	.5
71 years	4.0			.9	3.7	1.0

were categorized as truly Y-negative (fig. 1*a, b*). Cells missing both the faint hybridization signals and the major Y chromosomal signal were excluded from the evaluation.

Cells hybridized with the probes specific for chromosomes X, 1, or 17 showing only one hybridization signal of the same size as the signals in normal diploid cells were regarded to be hypoploid. If nuclei hybridized with the X-specific or autosomal probes showed only one signal obviously larger than the signals in neighboring cells, both signals were considered to be fused and the nuclei were categorized as disomic.

Statistical Analysis

The correlation (*r*) between donor age and frequency of chromosomal loss was calculated. The significance of the correlation (*r*) against the null-hypothesis was tested according to R. A. Fisher with the help of Student's *t*-distribution. A test for equality of the slopes was conducted (Draper and Smith 1981, p. 59 ff.).

Results

In order to determine the influence of culture conditions on aneuploidy rates, we first compared X- or Y-hypoploidy frequencies of resting lymphocytes isolated directly, by ficoll gradient centrifugation from six probands of different age groups to those obtained in lymphocytes cultured for 48 h and 72 h (table 1). Since no striking differences between resting and cultured cells were detected, the following analyses were carried out in 72-h–cultured cells, thus allowing the inclusion of healthy donors referred to the laboratory for chromosome analysis as relatives of affected individuals.

A total of 228,000 interphase nuclei from lymphocyte cultures of the 90 female and 138 male donors were analyzed by in situ hybridization for X and Y hypoploidy, respectively (figs. 1 and 2). Y-hyperploid nuclei, i.e., nuclei showing more than one distinct signal for the Y chromosome (fig. 1*c, d*) or more than two signals for the X chromosome were not scored in this study. Hybridization was either detected by avidin-fluorescein-isothiocyanate (avidin-FITC) (fig. 1) or by streptavidine-horseradish-peroxidase and DAB (fig. 2). In situ hybridization with chromosome 1– and chromosome 17–spe-

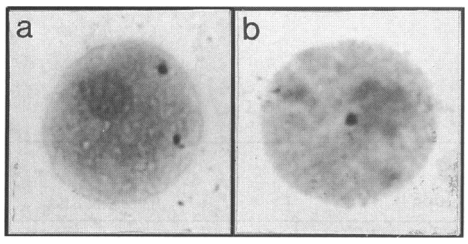

Figure 2 Interphase nuclei of a female proband hybridized in situ with biotinylated probe *pXBR*. *a*, Nucleus disomic for the X chromosome. *b*, Nucleus monosomic for the X chromosome. Signal detection was performed with streptavidine-horseradish-peroxidase.

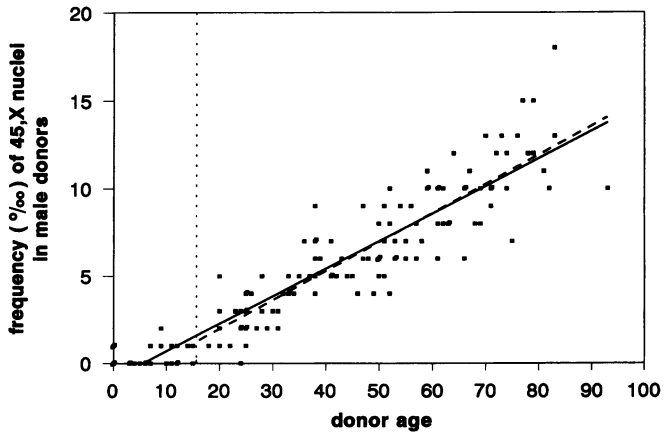

Figure 3 Frequency of Y chromosome loss in lymphocyte nuclei of male probands, plotted against donor age. Regression lines for the total group of probands (*bold line*) and for the men beyond age 15 years (*broken line*) are shown.

Table 2

Incidence of Y Chromosome Loss in Male Interphase Nuclei in the Different Age Groups Examined

Age Group (years)	No. of Probands	Average Age (years)	Nuclei (%) Lacking a Y-Specific Signal
0–5	10	1.5	.03
6–10	7	7.7	.05
11–15	8	12.8	.05
16–20	5	19.6	.24
21–25	13	24.3	.23
26–30	6	28.0	.32
31–35	8	32.8	.38
36–40	10	37.6	.63
41–45	7	42.4	.54
46–50	10	48.7	.60
51–55	12	52.7	.70
56–60	7	57.9	.87
61–65	9	62.0	.89
66–70	8	67.6	.95
71–75	8	72.6	1.04
76–80	5	77.8	1.34
81–85	4	82.3	1.30
>85	1	93.0	1.00

cific DNA probes on lymphocyte nuclei from one proband of each age group were performed in order to compare autosomal and gonosomal hypoploidy and to compare the hypoploidy rate for a large and a small autosome.

Y Chromosome Hypoploidy

The frequency of Y hypoploidy increased with age (fig. 3), as demonstrated by a highly positive linear correlation ($r = .9166$; $y = -0.8798 + 0.1575x$). The number of probands and the average frequency of Y-hypoploid cells per age group is given in table 2. The portion of Y-hypoploid nuclei was only 0.03% in children below age 5 years and remained relatively low until age 15 years (0.05%). In the 16–20-year-old probands the frequency rose to 0.24% and then continuously increased with age to a frequency of 1.34% in men age 76–80 years. The very low incidence of Y chromosome loss in boys up to the age of 15 years suggests that the 138 probands can be divided into two biological age groups: group A (prepuberty, age 0–15 years) being represented by 25 probands in the present study, and group B (age 16–93 years) being represented by 113 individuals. Regression analysis revealed two independent curves for both groups (fig. 3, table 3). Group A is represented by a flat line, indicating no correlation between Y chromosome loss and age ($r = .1487$; $y = 0.3252 + 0.0168x$). In contrast, the data for group B tightly fit to a line with a slope of 0.17, showing a very prominent age correlation ($r = .8740$; $y = -1.3697 + 0.1664x$).

X Chromosome Hypoploidy

The determination of X hypoploidy (fig. 2) presented an unexpected problem because of the possibility of signal fusion. In a number of normal cells the two hybridization signals of the X chromosomes were located very close to each other, appearing as only one signal. In these cases, the decision about hypoploidy was based on signal size and a second observer was consulted.

Interphase nuclei from 90 female donors age 1 wk to 91 years were analyzed. The overall incidence of X hypoploidy was distinctly higher than that determined for the Y chromosome, with much more variation among the various probands (e.g., 0.7%–3.4% X-hypoploid cells in the different women age 20–25 years, vs. 0%–0.5% Y-hypoploid cells in the males age 20–25 years) (fig. 4). The mean value of X monosomic cells increased from 1.58% in 0–5-year-old girls to 5.1% in a woman age 91 years (table 4).

For all probands, regression analysis showed a positive linear correlation between X chromosome loss and age ($r = .6712$; $y = 16.9258 + 0.3581x$). However, the probands can be divided into three biological age groups (group A, 0–12 years; group B, 13–51 years; and group C, 52–91 years) on the basis of sex-hormone function (12.8 years, mean age of menarche; and 51.2 years, mean age of menopause). A statistically significant correlation between X chromosome loss and age was obtained clearly for women of group C (table 5).

Group A ($n = 13$) is too small for statistical analysis. The slopes of the lines in groups B and C are not statisti-

Table 3

Statistical Data on Y Chromosome Loss in Males

Group	Age (years)	n	df	r	Slope	t	$t_{(df;.05;one\text{-}sided)}$	H_0[a]
All	0–93	138	136	.917	.16	26.74	1.65	–
A	0–15	25	23	.149	.02	.72	1.72	+
B	16–93	113	111	.874	.17	18.95	1.66	–

[a] A minus sign (–) = reject H_0, $r = 0$. A plus sign (+) = do not reject H_0, $r = 0$.

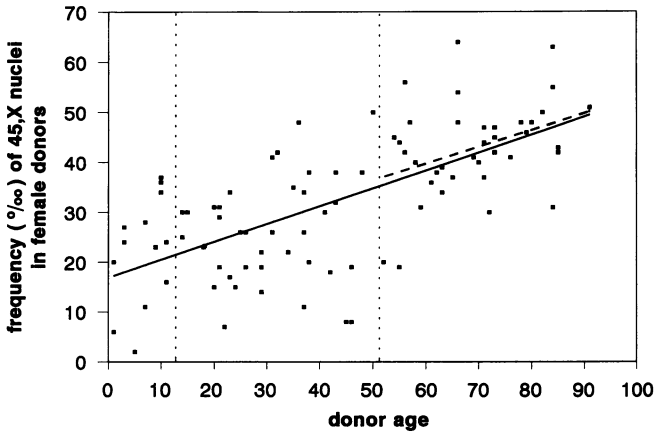

Figure 4 Frequency of X chromosome loss in lymphocyte nuclei of female probands, plotted against donor age. Regression lines for the total group of probands (*bold line*) and for females postmenopause (*broken line*) are shown.

cally different (P = .53). The slope in either group is not identical to 0 on the basis of P = .025; on the other hand, both regression lines are separated from each other (P = .000). This difference is also found if the female group is not divided at age 51 but at any age in the range of 40–60 years; that is, the present data only demonstrate that there is a change in the 50s, but it is not possible to determine the time more exactly.

Autosomal Hypoploidy

Chromosome 1 and chromosome 17 were chosen as autosomal controls, representing a large and a small autosome. Lymphocyte nuclei of 24 and 25 probands, respectively, were screened for chromosome 1 and 17 hypoploidy. As with the X chromosome, the problem of signal fusion had to be encountered, and in case of doubt a second observer was consulted. Since the size of the hybridization signals produced by both probes was almost identical, the risk of classifying these artifactual one-signal cells as monosomic was the same for both chromosomes.

No age correlation could be established for either autosome (fig. 5 and table 6). The incidence of chromosome 1 hypoploidy ranged from 0.7% in a 69-year-old proband, to 1.5% in a donor age 55 years, with an average of 1.2%. For chromosome 17 similar values were obtained (0.7%–1.4% hypoploid cells), with an average of 1% hypoploid cells.

Discussion

Chromosome loss during aging is a topic addressed in a number of previous investigations. However, all previous studies have employed metaphase analysis for the determination of chromosome loss. For technical reasons, metaphase analysis is restricted to a limited number of cells per proband and carries a significant risk of preparational aneuploidy. Since genuine aneuploidy is a relatively rare event, the risk of missing truly aneuploid cells by analysis of metaphase spreads is rather high. In situ hybridization with chromosome-specific DNA probes overcomes the disadvantages of metaphase analysis and permits screening of large numbers of interphase nuclei per donor.

The preliminary tests to evaluate a possible influence of short-term cell culture time on the incidence of sex chromosome loss showed no difference between resting lymphocytes and those being cultured for 2 or 3 d. Therefore, the rates obtained in cultured lymphocytes largely reflect the in vivo conditions. This conclusion is in agreement with the results from Richard et al. (1993), who found that autosomal but not gonosomal losses increased from 48-h to 72-h cultures.

Previous data on Y chromosome loss are nonuniform. Some authors found an age correlation (Jacobs et al. 1961, 1963; Court Brown et al. 1966; Fitzgerald and McEwan 1977; Galloway and Buckton 1978), whereas others did not detect a significant correlation between Y loss and donor age (Jarvik et al. 1976; Nowinski et al. 1990; Percy et al. 1993). This discrepancy, however, might be explained by the differing age distribution of probands: Jarvik et al. (1976) analyzed only a small sample of older donors (>80 years) over a 6-year interval, and the mean age of males studied by Nowinski et al. (1990) ranged from 9.8 to 31.8 years.

Table 4

Incidence of X Chromosome Loss in Female Interphase Nuclei in the Different Age Groups Examined

Age Group (years)	No. of Probands	Average Age (years)	Nuclei (%) Exhibiting a Sole X-specific Signal
0–5	5	2.6	1.58
6–10	6	8.8	2.82
11–15	5	13.0	2.50
16–20	4	19.0	2.30
21–25	8	22.5	2.23
26–30	5	27.8	2.00
31–35	5	32.6	3.32
36–40	6	37.2	2.95
41–45	5	42.8	2.52
46–50	4	47.5	3.13
51–55	4	54.0	3.20
56–60	5	57.2	4.35
61–65	5	62.8	3.68
66–70	5	67.5	4.94
71–75	7	72.0	4.17
76–80	4	78.3	4.58
81–85	6	84.0	4.73
>85	1	91.0	5.10

Table 5

Statistical Data on X Chromosome Loss in Females

Group	Age (years)	*n*	df	*r*	Slope	*t*	$t_{(df;.05;one\text{-}sided)}$	H_0[a]
All	0–91	90	88	.671	.36	8.49	1.66	–
A	0–12	13	11	.462	1.35	1.73	1.80	+
B	13–51	40	38	.166	.17	1.04	1.69	+
C	52–91	37	35	.362	.33	2.30	1.69	–

[a] A minus sign (–) = reject H_0, $r = 0$. A plus sign (+) = do not reject H_0, $r = 0$.

Our results show that loss of the Y chromosome does not continuously increase from childhood probands to old donors, as suggested by Jacobs et al. (1961, 1963), and that a significant age correlation is not limited to men older than 55 years (Galloway and Buckton 1978) or 65 years (Court Brown et al. 1966). Rather, our data show a highly significant correlation between Y chromosome loss and age in males older than 16 years. The onset of Y loss coincides with puberty in males and could possibly be explained by hormonal changes during that phase. In this context it would be of interest to analyze Y chromosome hypoploidy in other tissues, such as, e.g., cells of the testis. If hormonal changes in some way influence Y chromosome loss, this should also be detectable in the gonads. The increasing loss of the Y chromosome throughout adult life is reminiscent of the loss of the telomeric repeats that has been observed in human blood lymphocytes as a function of age (Vaziri et al. 1993). Owing to the relative paucity of genetic material, the human Y chromosome short arm may in fact be rendered more unstable by loss of telomeric DNA sequences.

An age correlation of X chromosome loss has been described by a number of authors. As with the Y chromosome, however, no comprehensive study has been performed to date analyzing 1,000 cells per donor as well as donors of all age groups. The overall incidence of X chromosome loss determined in the present study is two orders of magnitude higher than that determined for the Y chromosome. This difference could be due to a truly greater propensity of the X chromosome for mitotic errors, but it may also reflect some of the uncertainty in the definition of monosomic and disomic nuclei with overlapping hybridization signals. Double hybridization with two X-specific DNA-probes could largely overcome such problems.

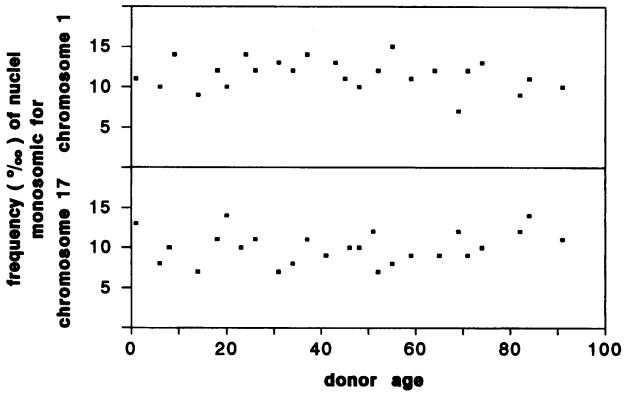

Figure 5 Frequency of chromosome 1 and chromosome 17 loss in lymphocyte nuclei of different probands, plotted against donor age.

The overall correlation between X chromosome loss and age is not as strong as that for Y chromosome loss. If the female probands are (arbitrarily) subdivided into three reproductive age groups (<12.8 years, 12.8–51.2 years, and >51.2 years) no significant age correlation of X chromosome loss is found prior to menarche and menopause. Only women older than 51 years show a distinct age correlation. Horsman et al. (1987) suggested that in vivo physiological variation of hormone concentration may play a role in X chromosome aneuploidy. Moreover, the estrogen/progesterone balance of the donor at the time of blood collection may influence the rate of lymphocyte proliferation and mitotic behavior (Wheeler et al. 1986). Our data clearly support the notion that sex chromosome loss in the female increases significantly only beyond the reproductive age.

The data obtained for the autosome numbers 1 and 17 confirm the finding that loss of autosomes is not correlated with age (Ford and Russell 1985; Nowinski et al. 1990; Richard et al. 1993). In our study, nuclei monosomic for chromosome 1 and chromosome 17 were found with nearly identical frequencies (1.2% and 1%). This observation is in contrast to that made by several authors who reported that incidence of autosome loss is inverse to chromosome length (Neurath et al. 1970; Wenger et al. 1984; Percy et al. 1993; Richard et al. 1993). This discrepancy is most likely due to the different methods of analysis (metaphase vs. interphase nuclei). The fact that chromosomes 1 and 17 show similar rates of aneuploidy suggests that large and small

Table 6

Statistical Data on Autosome Loss

Chromosome	Donor Age (years)	n	df	r	Slope	t	$t_{(df;.05;one\text{-}sided)}$	H_0[a]
1	1–91	24	22	−.187	−.01	.89	1.72	+
17	1–91	25	23	.149	.01	.72	1.71	+

[a] A plus sign (+) = do not reject H_0, $r = 0$.

autosomes are lost with the same frequency in interphase nuclei. In metaphase analyses, preferential loss of smaller chromosomes might be due to preparational artifacts.

As to the likely mechanisms of age-related chromosome loss in lymphocyte nuclei, Ford and Russell (1985) suggested that chromosome displacement leading to anaphase lagging accounts for autosomal chromosome errors. In aged women, chromosomes tend to lose their Cd-positive material, leading to impairment of centromere function (Nagakome et al. 1984). Premature division of the sex chromosome centromere (PCD) increases the risk of nondisjunction causing aneuploidy of the X chromosome and possibly also of the Y chromosome (Fitzgerald et al. 1975; Fitzgerald and McEwan 1977). Since PCD of the X chromosome is lower in males than in females, it is likely that the inactive X chromosome is preferentially prone to PCD. Abruzzo et al. (1985) confirmed this notion, showing that the inactive X chromosome is missing in most X monosomic cells.

Recent studies show that the number of micronuclei also increases with age in human blood lymphocytes (Fenech and Morley 1985; Ganguly 1993). Moreover, in situ hybridization with X- and Y-specific DNA probes proves that a considerable portion of such micronuclei contain X or Y chromosomes (Hando et al. 1994; Guttenbach et al. 1994).

In summary, the present investigation shows that sex chromosome loss but not autosome loss is strongly correlated with age. Loss of the second X chromosome in females is much more frequent than loss of the Y in males, but this loss shows age dependency only after menopause. Autosomal hypoploidy is not age dependent. Loss of autosomes is likely to lead to cell death, since autosomes contain and express genes important for cell survival, which is not the case for the genetically inactive X and the largely heterochromatic Y chromosome.

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