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Chromosome Elimination in Micronuclei: A Common Cause of Hypoploidy

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Summary

An excess of hypoploid cells has repeatedly been reported in studies of aneuploidy and has often been attributed to technical artifact. We have examined at least 200 anaphase or early-telophase cells from each of 28 normal women and found that chromosome or chromatid lagging occurs in an average of 2.43% of cells. In a separate study, we have examined the frequency of micronuclei in cytochalasin B–arrested, binucleate cells and shown that a similar frequency of cells (1.6%) contain one or more micronuclei. Using in situ hybridization of an alpha centromeric probe (αR_1), which hybridizes to 9 of the 22 human autosomes, we were able to infer that most, if not all, of the micronuclei contain whole chromosomes or chromatids. Since the loss of a chromosome by lagging will induce hypoploid daughter nuclei (two where a chromosome is lost and one where a chromatid is lost), we conclude that lagging is a major mechanism for chromosome loss in human lymphocyte cultures. This loss occurs in the cells of normal individuals under control conditions.

Chromosome Elimination in Micronuclei: a Common Cause of Hypoploidy

All studies of aneuploidy in human lymphocytes have found a significant excess in the frequency of hypoploid cells over hyperploid cells (e.g., 110 hypoploid to 13 hyperploid in a total of 3,396 lymphocytes [Fitzgerald and McEwan 1977]). These results are incompatible with the expected one loss to one gain predicted by nondisjunction, and the excess of hypoploid cells has often been regarded as a technical artifact. When the rates of loss of specific chromosomes have been examined, chromosomes are found to be lost in a consistent manner. Small chromosomes are lost much more frequently than large chromosomes (Brown et al. 1983). Two different explanations have been put forward to explain the data. First, the hypoploidy might reflect some specific technical problem, as suggested by Brown et al. (1983). Alternatively, the hypoploid cells could be the product of a cell division where a chromosome or its chromatids fails to move into either daughter cell and is subsequently eliminated. Such divisions involving the loss of whole chromosomes would create two hypoploid products, as suggested by Ford and Roberts (1983). Divisions involving the loss of one of a pair of sister chromatids would create one diploid and one hypoploid product.

The mechanism by which chromosomes or chromatids might be eliminated from each of the daughter cells has not yet been defined, but it is likely to involve lagging at anaphase. We suggest that chromosomes that fail to attach to the spindle, probably those displaced at metaphase, are predisposed to "lag" at the metaphase plate after the other chromatids have moved to the spindle poles. The fate of the lagging chromosomes or chromatids might be micronucleation at the time of nuclear membrane formation. In his studies of male meiosis in the grasshopper *Attractomorpha similis*, Peters (1981) observed that, in animals that were commonly trisomic, lagging univalents were eliminated as micronuclei with a high frequency. Lagging here appeared to result from inappropriate orientation of the univalent. Although the etiology of lagging in mitosis is probably different from that in meiosis, the outcome of lagging may well be the same.

Hypoploidy is reported in lymphocyte cultures in fre-

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quencies from 3.2% (Fitzgerald and McEwan 1977) to 11.8% (Brown et al. 1983). If chromosome elimination is to account for some or all of this, it is necessary to demonstrate both that lagging and micronuclei occur and that they occur at appropriate frequencies. Two different studies were conducted.

1. *Measurement of the Frequency of Anaphase Lag in Lymphocyte Mitosis*

Cells at anaphase were examined to determine whether whole chromosomes or chromatids remained at the metaphase plate after the separation and movement of the other chromatids. If so, then with what frequency did this occur?

2. *Frequency and Nature of Micronuclei in Cytochalasin B–Induced Binucleate Cells*

Cytokinesis was inhibited in dividing lymphocytes by the addition of cytochalasin B to the cultures. With this technique, it is possible to observe the products of a single cell division within the one cytoplasm and to assess the rate of micronucleus formation in a cell division.

In situ hybridization with an αR_1 probe was used to detect the presence of pericentromeric DNA in micronuclei and thus infer the presence of whole chromosomes in the micronuclei. We assume that no acentric fragments are generated by breakage between the centric heterochromatin and the centromere. To conduct the experiments we required a probe that would bind specifically to the centromeric regions of several or all of the human chromosomes. The αR_1 probe was selected because it hybridized to several of the human chromosomes and was a probe with which we had had considerable experience in our laboratory.

The frequency of cells with labeled micronuclei was determined. From this, the frequency of micronuclei with whole chromosomes or chromatids could be calculated and compared with the anaphase data.

The data obtained in both studies were consistent with the proposition that chromosomes or chromatids that fail to attach to the spindle at metaphase lag at anaphase and are eliminated in micronuclei. The frequency of the elimination (lagging and micronucleation) was sufficient to account for most of the hypoploidy observed in human lymphocyte cultures.

Material and Methods

All studies were performed on cultures of PHA-stimulated blood lymphocytes. Normal volunteers were used in each experiment.

1. *Anaphase Studies*

Whole blood cultures were established, and anaphase frequency was increased in them by the technique described by Ford and Congedi (1987). Actively growing lymphocyte cultures were placed in a 37 C water bath. At about 72 h after culture initiation, the media was gently pipetted off, without disturbing the cells. Three milliliters of 0.075 M KCl (prewarmed to 37 C) were then added to the culture tubes for 5 min. After 5 min, 2 ml of fix (3:1, methanol:acetic acid) were rapidly squirted into the hypotonic mix and the tubes were plunged into ice. After 10 min on the ice, 5 ml of fresh fix were added and the cells were completely suspended before centrifugation. Further fixation, spreading, and air-drying were then performed as usual for lymphocyte cytogenetics. The slides were stained for 3 min in 5% Giemsa (in phosphate buffer, pH 6.8). This technique ensures that about 30% of the dividing-cell population is observed in anaphase.

Chromosomes or chromatids were scored as lagging if they were either completely separated from the rest of the chromatids at anaphase A or remained in the region of the metaphase plate at anaphase B/early telophase (fig. 1). Early telophase cells were those in which cytokinesis was incomplete. Cells showing distortion of the anaphase configuration, which could lead to misinterpretations, were excluded from the scoring.

2. *Micronuclei Studies in Binucleate Cells*

Induction of binucleate cells.—Binucleate cells were induced for the assessment of micronuclei by using a modification of the method of Fenech and Morley (1985). Lymphocytes were isolated from whole blood by centrifugation at 1,500 *g* in a Ficoll-Paque gradient (Pharmacia). These were then cultured at a concentration of about 1 million/ml in RPMI medium with 20% FCS and 0.2% PHA (Wellcome HA15). After 68h, cytochalasin B (Sigma) (made up in a stock solution of 2 mg/ml in dimethyl-sulfoxide [DMSO]) was added, to a final concentration of 3 μg/ml. After 28 h the cells were centrifuged and 7 ml of 0.075 M KCl was added as hypotonic for 20 min. Three milliliters of fixative (3:1, methanol:acetic acid) was added to the hypotonic, and the tubes were centrifuged. The cells were fixed in 10 ml of fixative for 10 min, followed by two further changes of fixative. The total period of fixation was about 2 h. The cells were then dropped gently onto clean dry slides and allowed to air-dry.

Identification of micronuclei.—The slides were stained in 5% Giemsa (phosphate buffered, pH6.8) and scanned for micronuclei with an oil-immersion 100× high-power objective. The proportion of positive cells was noted,

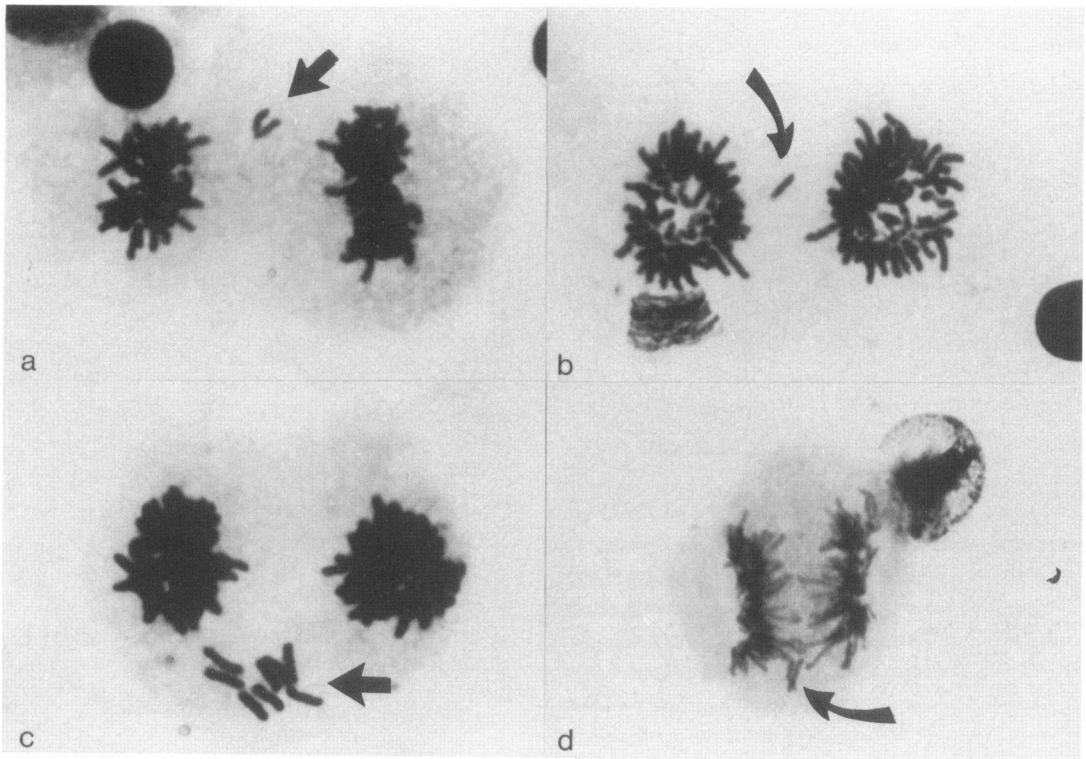

Figure 1 Examples of chromosome and chromatid lagging in anaphase and early telophase. 1*a* shows a single chromosome lagging, whereas 1*c* shows four chromosomes lagging. In each of these, the chromatids have separated from each other but have not moved toward the poles. In 1*b* a single chromatid is lagging at anaphase. Presumably the other chromatid has moved to one of the poles. 1*d* shows one chromosome lagging at early anaphase. Here, there is no suggestion of chromatid separation.

and the location of each was recorded with the use of an England Finder. Each micronucleus-positive, binucleate cell was then photographed. The cells were destained in 80% ethanol and air-dried.

In situ hybridization technique.—The slides were denatured with 70% formamide in 2 × SCC at 70 C for 2 min, dehydrated as before, and air-dried. The slides were hybridized with 100 ng of nick-translated αR_1 probe (activity, 5.3 × 10 dpm/μg labeled with tritiated deoxyguanosine 5′-trisphosphate) in a 1 ml hybridization solution which contained 0.1 g of dextran sulfate 500 and 50 μg of salmon sperm DNA. The incubation was allowed to proceed in a moist chamber overnight at 37 C.

The slides were washed under stringent conditions (3 washes, 50% formamide in 2 × SSC at 40 C for 2 min each), dehydrated, and air-dried. They were dipped in Ilford L4 nuclear emulsion and exposed at 4 C for 4–7 wk. The slides were developed in Kodak D19 developer, rinsed and fixed in Ilford Hypam fixer, then washed in six changes of distilled water over 1 h. The slides were restained in 10% Giemsa for 5 min. The previously identified micronuclei were relocated and photographed.

Method of indirect identification of chromosomes in micronuclei.—Metaphases that were prepared from cells of the subject used for the production of binucleate cells were used to determine the specificity of chromosomal labeling with αR_1. Photographs of 17 metaphase spreads were taken, and, with graph paper, the area covered by grains was recorded for each chromosome. For each cell, the area covered by grains for a certain chromosome pair was expressed as a percentage of the total area covered by grains in that cell. This percentage for the chromosome pair was then averaged for the 17 cells, and the standard errors were calculated (table 1).

The αR_1 probe hybridizes with the centromeric region of nine of the 23 pairs of human chromosomes. The other chromosomes show negligible labeling. There is considerable between-cell variation in the specificity of chromosome labeling, and figure 2 shows a cell in which only five chromosomes are heavily labeled. The detailed analysis of this hybridization distribution and

Table 1

Mean Percentage Grain Area Occupied by a Chromosome Pair

Chromosome Pair	Mean	Standard Error
1	26.9	1.44
5	22.1	2.07
19	19.3	1.06
16	11.7	0.86
7	5.9	0.95
10	5.1	0.83
3	3.8	0.75
12	3.4	0.61
6	1.7	0.51
Total	100.0	

its applicability to polymorphisms will be discussed elsewhere (paper in preparation). To adjust for the between-cell variability and to gain more accuracy in the micronucleus study, the 17 metaphase cells were classified as being of low (seven), moderate (five), or high (five) labeling density. The absolute areas occupied by grains (rather than the relative area as used above) were used to calculate the mean areas occupied by grains for each chromosome in each group. These areas are shown in table 2.

A micronucleus was regarded as positive if it contained two or more grains lying no further apart than the diameter of a single grain. By the graph-paper method, the diameter of a single grain was 1.0 mm, and thus a positive cell had a minimum labeled area of 2.0 mm^2. In a few cases a negative micronucleus could have been scored incorrectly as positive. For each binucleate cell that contained a micronucleus with grains, the areas of grains in the micronucleus and in the two nuclei was determined. The area of grains in the micronucleus was then expressed as a percentage of the total. Again, the cells were classified as being of low, moderate, or high labeling density.

At the end of telophase prior to G1, positive micronuclei would contain either whole chromosomes or chromatids whereas nuclei would contain chromatids. Provided the DNA in a micronucleus is replicated at the same rate and at the same time as the DNA in the nuclei in that cell, then the DNA content relationship between the micronucleus and the nuclei will be constant and independent of the stage of the cell cycle (i.e., G1, S, or G2). Thus the expected percentage of area covered by grains in a micronucleus, compared with the total area covered by grains for the whole binucleate cell and micronucleus, can be estimated. Where the micronucleus contains a chromosome, the area is half that for the particular chromosome pair. Where the micronucleus contains a chromatid, the area is one-quarter of that for the particular chromosome pair.

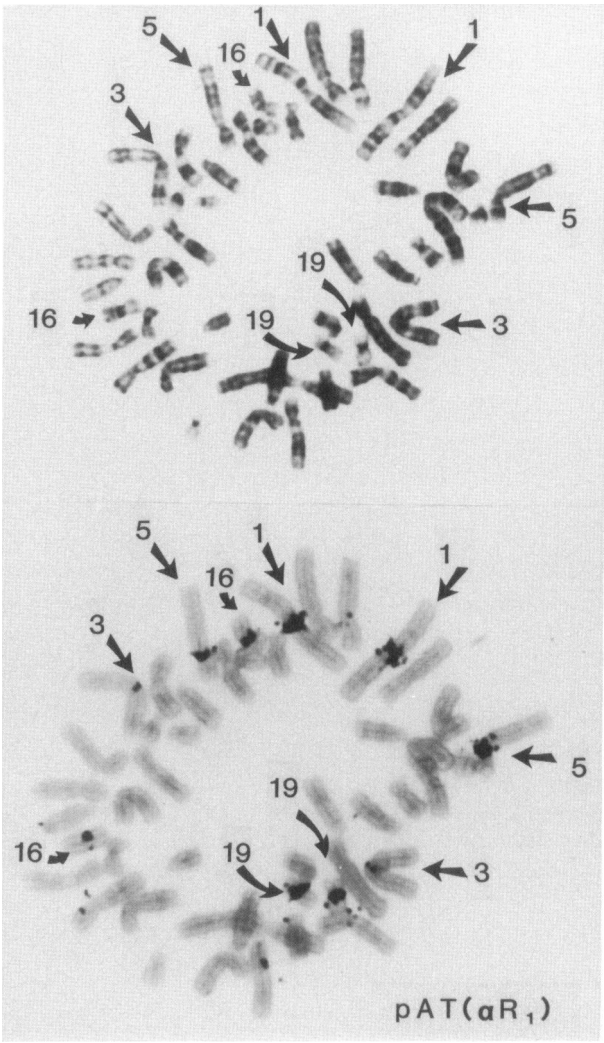

Figure 2 A metaphase preparation photographed first with Giemsa banding (*top*) and then after in situ hybridization with the αR_1 probe (*bottom*). The five chromosomes showing the highest levels of labeling in this cell are identified. The other chromosomes labeled by αR_1 (i.e., 7, 10, 12, and 16) and either unlabeled or weakly labeled here.

The variability between cells and the constant minimum acceptance for a positive micronucleus means that cells of different labeling density have different sensitivity (i.e, lower-density cells will not detect the presence of all chromosomes). The sensitivities shown in table 3 have been estimated by comparing the data in table 2 with the 2.0-mm^2 grain area cutoff point.

Table 2

Grain Area Occupied by Chromosomes at Different Labeling Intensities

	Chromosome Pair								
Intensity	1	5	19	16	7	10	3	12	6
Low:									
Mean (mm^2)	59.7	43.9	37.1	22.6	6.0	6.9	3.9	3.7	0.7
Standard error	4.1	4.0	3.1	2.5	2.3	2.6	1.4	1.6	0.4
Moderate:									
Mean (mm^2)	75.6	74.6	62.8	34.0	17.4	14.4	9.8	10.4	7.8
Standard error	6.6	18.2	5.1	6.8	3.1	5.2	5.2	3.7	3.7
High:									
Mean (mm^2)	107.2	85.0	83.6	57.8	52.2	42.6	36.6	29.0	14.0
Standard error	5.2	1.8	8.4	5.8	7.3	4.7	5.0	4.8	4.2

Results

Anaphase Lagging

A minimum of 200 anaphases or very early telophases were analyzed in each of 28 normal women whose ages ranged from 25 to 55 (mean 40.04 ± 8.13) years. All these individuals were known to have reproduced normally and to have had no miscarriages. Lagging was seen in all cultures and was observed with frequencies that ranged from 1.0% to 5.3%. The mean frequency was 2.43%, SD ± 1.22. In 64% of cells showing lagging, chromatids rather than chromosomes were involved. Examples of lagging are shown in figure 1.

Where chromosomes were lagging, the chromatids of the lagging chromosomes had usually separated from each other (figs. 1*a*, 1*c*). In only a few cases, such as that shown in figure 1*d*, the chromatids remained together. A single-chromosome origin of the lagging chromatid or chromosome was found in 80% of cells (figs. 1*a*, 1*b*, 1*c*).

Micronuclei in Binucleate Cells

This experiment used the cells of a male subject aged 33 years. Two blood cultures were made 1 wk apart. The frequencies of micronuclei found were not significantly different between the two experiments, so the results were pooled. A total of 1,800 binucleate cells were screened to determine the frequency of cells with a micronucleus. This was found to be 1.6%.

One hundred thirteen cells, containing 124 micronuclei, were photographed for use in the in situ hybrid-

Table 3

Sensitivity of Chromosome Detection at Different Intensities of Label

	Chromosome Pair								
Labeling	1	5	19	16	7	10	3	12	6
Low:									
Chromatid	X	X	X	X	–	–	–	–	–
Chromosome	X	X	X	X	X	X	–	–	–
Moderate:									
Chromatid	X	X	X	X	X	X	X	X	–
Chromosome	X	X	X	X	X	X	X	X	X
High:									
Chromatid	X	X	X	X	X	X	X	X	X
Chromosome	X	X	X	X	X	X	X	X	X

Note.—X = will be detected if present; – = will not be detected.

ization study. Nine (7.96%) of the 113 cells contained more than one micronucleus, seven cells had two micronuclei, and two had three micronuclei. Forty of the micronuclei contained grains of photographic emulsion exceeding 2.0 mm^2 (i.e., were considered positive). Cells, with and without grains, both before and after hybridization, are shown in figure 3. The areas occupied by grains in the positive cells were determined by the method described. The results of this analysis are shown in table 4.

The results in table 4 indicate that there is often considerable variability between the areas of grains in the two nuclei in a binucleate cell. This inequality is not accounted for by asymmetric loss, as the differences are often very large and could not be accounted for by nondisjunction of the chromosome with even the highest degree of labeling. Further, these differences can be seen both in binucleate cells with micronuclei and in those without micronuclei. The differences may be due to either the imprecision of the technique or asynchronous replication of the nuclei (Ridler and Smith 1968). For this reason, this technique is unlikely to provide accurate identification of individual chromosomes in micronuclei.

Of 124 micronuclei, 40 were considered to be labeled. This is a frequency of .323. Thirteen of the cells carrying micronuclei were judged to be low labeling, 16 were moderate labeling, and 11 were high labeling. Differences in sensitivity (table 3) were taken into account in the calculation of expected frequencies.

If chromosomes are incorporated in micronuclei on a random basis, labeled chromosomes or chromatids would be expected to be found with a frequency of .297. A χ^2 test ($\chi^2 = 0.002$) shows that the observed result is not significantly different from this expected frequency. Alternatively, chromosomes or chromatids might be found in micronuclei at frequencies determined by their relative rates of displacement (Ford and Lester 1982). The expected frequency of positive micronuclei, calculated from the relative rates of displacement (also adjusted for cell labeling intensity and sensitivity), is .302. A χ^2 test here gives a value of 0.001, which is not significant.

Discussion

Examination of lymphocytes at late anaphase/early telophase demonstrated a low frequency of lagging in all the individuals tested. This occurred under normal culture conditions without the addition of any inducing chemicals. Five of the 28 women studied had lagging in only 1% of their cells, whereas two had lagging in more than 5% of their cells. The others had intermediate frequencies, and the mean was 2.43%. It is not known at this stage whether individuals show consistent behavior on different occasions. In almost all of the cells with chromosome lagging, the chromosomes had disjoined at the centromeres but neither chromatid had moved poleward. Presumably, neither kinetochore had made an appropriate attachment to the spindle. In cells where only one chromatid was lagging, it seems likely that one kinetochore was able to make an attachment to the spindle, so as to allow that chromatid to segregate into a daughter cell. In many cases the lagging chromosomes and chromatids appeared to be pushed sideways with the formation of the cell plate (see, e.g., figs. 1*a*, 1*c*, 1*d*).

Micronuclei studies were performed in only one male subject. They were found with a frequency of 1.6%, which is of the same order as the frequencies detected for anaphase lagging. The finding of alpha centromeric sequences in the micronuclei indicates that the micronuclei contain chromosomes or chromatids with centromeres. This concept is supported by other studies, including the positive staining of micronuclei with anticentromeric antibody (Rudd et al. 1986) and fusion experiments (Viaggi et al. 1987). Such results show that, under control conditions, micronuclei contain whole chromosomes or chromatids.

The data strongly support the proposal that chromosomes and chromatids that lag at anaphase can become micronuclei. The two daughter cells resulting from an elimination of a whole chromosome would both be hypoploid. In the cases where a chromatid is eliminated, one daughter cell would be diploid and the other hypoploid. Provided that hypoploid cells are viable in culture, then the 2% (approximately) frequency of elimination in each cell division could account for a considerable proportion of the hypoploidy observed in human lymphocyte cultures.

Possible Relationship to Chromosome Displacement

The model for chromosome displacement (Ford and Roberts 1983) suggests that two of the possible fates for chromosomes displaced at metaphase are "retrieval" and "chromosome elimination." Since displaced chromosomes are probably not attached to the spindle at metaphase (Correll and Ford 1987), if they failed to make new spindle attachments, they would be likely to lag at anaphase. Chromatid lag most likely reflects partial retrieval, with only one kinetochore making an appropriate attachment to the spindle. Both the fre-

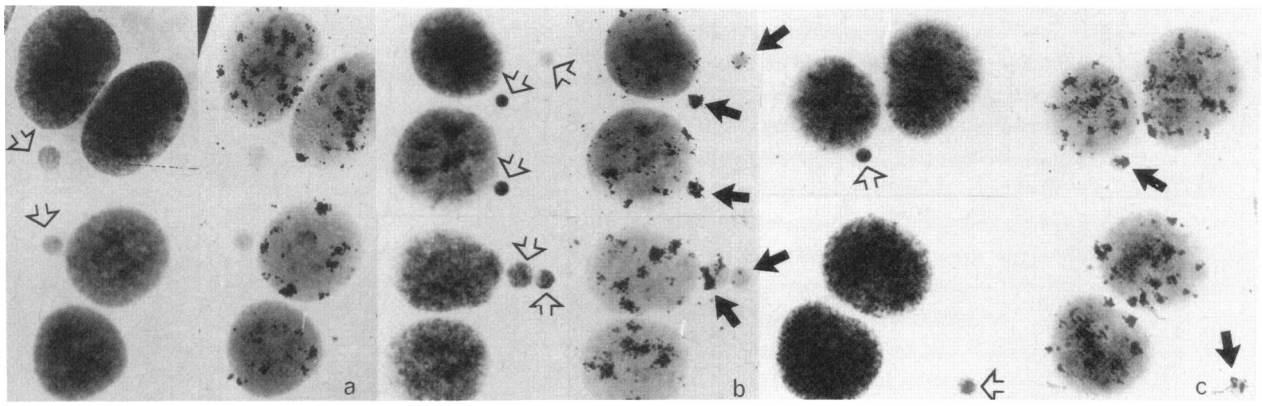

Figure 3 Binucleate cells with micronuclei, before and after hybridization with the αR_1 probe. In *3a* neither of the micronuclei is labeled, whereas in *3b* and *3c* micronuclei are labeled. *3b* shows cells that contain more than one micronucleus. Unlabeled micronuclei are indicated by unfilled arrows, labeled micronuclei by filled arrows.

quency of metaphases judged as showing displacement and the frequency of displaced chromosomes per metaphase (84.58% and 2.34%, respectively [Ford and Roberts 1984]) are significantly higher, respectively, than the frequency of cells showing lagging and the number of lagging chromosomes per cell (2.43% and 1.1%, respectively). If displacement usually precedes anaphase lag, then either the somewhat arbitrary cutoff points for the definition of displacement are too generous or retrieval occurs in most cases.

The relative involvement of chromosomes in displacement has been shown previously to be very similar to the relative involvement of chromosomes in hypoploidy. It is likely that displacement is an early step in the elimination process, but further studies, using chromosome-specific probes, are required to substantiate this.

Common Mechanism of Chromosome Loss and Hypoploidy in Human Lymphocytes

From these studies, we conclude that many of the

Table 4

Percentage Grain Areas Occupied by Nuclei and Micronuclei in a Common Cytoplasm

Cell	Nucleus 1	Nucleus 2	Micronuclei	Cell	Nucleus 1	Nucleus 2	Micronuclei
T12	56.0	38.4	5.7	K40	52.4	45.9	1.6
R34	52.3	37.8	9.9	M32.....	49.8	48.6	1.5
H38	55.6	43.0	1.4	L37	60.6	38.5	0.9
Q14	47.6	46.9	5.6	Y33	51.3	47.8	0.9
S30	47.0	44.2	8.8	K30	63.2	34.5	2.3
N13	57.2	38.5	4.4	Q32	59.4	38.9	1.7
T40	50.9	39.4	9.7	J12.....	52.7	43.7	3.6
U34	51.0	47.0	2.0	P39	58.1	40.3	1.6
N37a	38.1	40.7	10.6	Q15	53.3	43.3	3.4
N37b	38.1	40.7	10.7	S29	50.0	48.7	1.3
P30	50.2	49.1	0.8	T30	55.0	42.9	2.1
L28a	49.5	47.5	3.0	Q35	52.5	47.1	0.4
O27.....	61.2	35.3	3.5	S10	54.5	44.7	0.9
T25	64.4	34.8	0.8	R32	49.9	48.6	1.5
X31a	57.1	33.8	8.0	Q20	59.0	40.4	0.5
X31b	57.1	33.8	1.0	L28b	48.0	44.1	7.9
M40.....	56.7	39.6	3.7	S32	53.9	41.4	4.7
V27	60.0	38.5	1.5	W21.....	73.2	19.9	6.9
L31	51.8	45.7	2.5	G30	55.7	38.8	5.6
F39	46.8	41.1	12.1	G17	53.1	44.1	2.9

micronuclei seen in human lymphocytes contain whole chromosomes, rather than acentric fragments. Thus we suggest that a common mechanism of chromosome loss occurs in untreated cells cultured from the cells of normal individuals. This mechanism may involve chromosome displacement at metaphase and is seen as chromosome and chromatid lag in late anaphase/early telophase and as micronucleation at later stages. The result of each of these abnormal cell divisions is one or two hypoploid daughter cells, respectively. The frequency of chromosome elimination would account for much of the hypoploidy observed in lymphocyte cultures, provided the hypoploid cells are viable under these culture conditions.

In human cytogenetics, nondisjunction is widely assumed to be responsible for the generation of aneuploidy, both in meiotic errors which give rise to constitutional trisomy and in mitotic errors, which give rise to mosaicism. In his review of nondisjunction, C. E. Ford (1981) questions this assumption, on the basis that nondisjunction has not been directly observed in any human cells. He proposes that chromosome lagging might be an "entirely plausible" alternative, although, as he points out, it is similarly unknown, partly because "observations at anaphase in mammalian material are rare." The development of a technique to observe reasonable numbers of lagging chromosomes and chromatids at anaphase has enabled us to estimate the frequency of mitotic lagging in human lymphocytes. Our use of a DNA probe specific to several pericentromeric regions provides us with data on the frequency of certain centromeric regions (and presumably whole chromosomes or chromatids) within lymphocytic micronuclei. These two frequencies are similar, and so we suggest that lagging is a major mechanism for chromosome and chromatid loss within this material.

Acknowledgments

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