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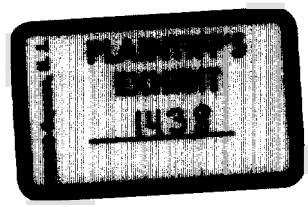
Sequence of centromere separation: kinetochore formation in induced laggards and micronuclei



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Mouse L-cells were treated with a bis-benzimidazole derivative (Hoechst 33258), caffeine and bleomycin in order to study genesis of laggards and micronuclei and formation of kinetochores as revealed by antikinetochore antibody staining. Apparently, the Hoechst 33258-induced decondensation experienced by the A:T-rich pericentric heterochromatin does not extend into the centromeric region and does not affect formation, physical appearance or function of kinetochores. The laggards induced by Hoechst 33258 are generally whole chromosome laggards which have antikinetochore antibody binding sites. These kinetochore-carrying laggards were seen to lie outside the spindle region in cells untreated with spindle inhibitors or hypotonic and stained differentially for spindle and chromosomes. Some micronuclei did not show kinetochore dots indicating their origin in acentric chromosome fragments. When cells were treated with caffeine or bleomycin, both types of micronuclei, namely those carrying kinetochores and those generated by acentric fragments, were seen. These results are interesting in that caffeine prevents the rejoining of chromosome breaks and one would expect only kinetochore-less micronuclei in caffeine-treated cells. It may mean that caffeine also induces aneuploidy. The L-cells carry minichromosomes which are no more than a pair of kinetochore dots. Such chromosomes, though detectable by antikinetochore antibody staining, may be missed in routine, acid-fixed, Giemsa-stained preparations.

Introduction

It is generally recognized that aneuploidy can be induced by the bis-benzimidazole derivative, Hoechst 33258 (Vig and Swearngin, 1985). It is, however, not known: (i) if such lack of equational segregation has anything to do with decondensation of pericentric heterochromatin which Hoechst 33258 is capable of inducing in A:T-rich DNA in the G_2 phase (Goitein *et al.*, 1984); (ii) if such aneuploidy results from the so-called out-of-phase centromere separation (Vig and Swearngin, 1985), or (iii) if Hoechst 33258 can induce laggards which can result in genesis of aneuploidy. Alternatively, it is possible that kinetochore proteins are absent from centromeric regions in some chromosomes treated with Hoechst 33258 (Lica *et al.*, 1982). The latter. if true, may result from a decondensation effect extending well into the centromeric region tlanked by heterochromatin and, consequently, modifying the spindle binding kinetochore proteins.

Caffeine and bleomycin, on the other hand, have no effect on the physical structure of pericentric heterochromatin. Bleomycin is a typical radiomimetic agent which causes chromosome aberrations and permits rejoining of broken ends. It induces aberrations in any part of the cell cycle (Vig and Lewis, 1978). Caffeine, however, is capable of causing chromosome aberrations in cells only in the S-phase. It does not permit the rejoining of broken ends when mammalian cells are cultured at 37°C (Kihlman, 1977). Whereas caffeine-treated cells do show chromosome loss, chromosome gain in mammalian cells treated with caffeine has been a point of dispute (Bond and Chandley, 1983).

The original purpose of this study was to find out if Hoechst 33258-induced aneuploidy results from laggards which lack kinetochore formation at the centromeric region. This can be done by the application of antikinetochore antibody found in the sera of certain scleroderma patients and looking for the presence of kinetochore proteins in the lagging chromosomes and micronuclei which result from Hoechst 33258 treatment. Bleomycin was included for a comparison. If chromosome aberrations, like bridges resulting from translocations cause micronuclei formation, these micronuclei should show kinetochore antibody binding sites. On the other hand, caffeine-induced micronuclei should have their origin primarily in acentric fragments and should be free of kinetochores.

Materials and methods

A subline of mouse L-cells was used in these studies. These cells were originally obtained from American Type Culture Collection (ATCC CCL 9.1, NTC clone 1469). The modal chromosome number in our subculture is 63 (range 58–67). These cells have between 13 and 16 dicentrics resulting from Robertsonian fusions of acrocentric chromosomes and one characteristic marker which has been interpreted to be an octacentric (Vig, 1984; Vig *et al.*, 1985; Rattner and Lin. 1985). In some studies a cell line derived from pig (*Sus demesticus*) kidney was also used. These cells lines were used because of their ready availability in our laboratory. The cells were grown in McCoy's 5A medium supplemented with 15% fetal calf serum and 1000 units/ml of Penn-strep mixture.

In order to extend the A:T rich heterochromatin, the cells were treated with 35 μg/ml Hoechst 33258 for varying periods ranging from 2 to 35 h. To study if cells treated with Hoechst 33258 for 2 h were in S or post-S-phase. the cultures were fed simultaneously with [^{3}H]TdR at a dose of 5 μCi/ml (sp. act. 55 mCi/mM).

In order to study the micronuclei induced by caffeine and bleomycin, the cultures were treated with a final concentration of 0.3% caffeine or 0.001% bleomyicn for 2 h. Samples were taken at various intervals to find out if chromosome aberrations were induced with these concentrations. This was done only to ensure that micronuclei would be induced by the time the cells traverse through a second cell cycle. The purpose was not to correlate the frequency of aberrations with that of micronuclei in various subpopulations or at various intervals. Hence, no effort was made to correlate these parameters. For cells treated with caffeine a 12-h recovery provided ample evidence of chromosome aberrations having occurred; for bleomycin it was 24 h. For the study of micronuclei an additional 12-h recovery was provided for each chemical.

The cells were harvested by using Colcemid (0.01 pg/ml) for the last 2 h of culture. For routine preparations, the cells were exposed to 12 min hypotonic (0.075 M KCl) and fixed in a 3:1 mixture of methanol:acetic acid. In experiments designed to study the position of laggards the cells were collected by using mitotic shake-off without application of Colcemid or subsequent hypotonic treatment. Preparations were made by using sequential double staining with safanin O and brilliant blue R for visualizing spindle and Giemsa for chromosomes following the technique of Wissinger *et al.* (1980). Autoradiographs were prepared 10 days after harvesting the cells labeled with [^{3}H]TdR.

The kinetochore proteins were tagged with the antikinetochore antibody found in the serum of scleroderma (var. CREST) patients. Kinetochore protein–antikinetochore antibody complex was visualized under a fluorescent microscope by using goat anti-human IgG (Davis Antibodies. Inc). The details of the technique were similar to those used by Brenner *et al.* (1981) except that chromosomal DNA was stained with propidium iodide (Zinkowski *et al.*, 1986).

Results

Hoechst 33258-treated cells

Undercondensation of ***heterochromatin.*** Mouse heterochromatin in our cell population expressed undercondensation when treated with Hoechst 33258 for at least 1 h (Figure 1); longer treatment resulted in extensive decondensation. When cells labeled with tritium were prepared for autoradiography. the metaphase chromosomes showed no sign of label. Enough label was, however, present in cells in interphase. This observation agrees with that of Goitein ***et al.*** ***(1984)*** in that undercondensation of mouse chromosomes can take place during G_2.

Table I. Frequency of laggard chromosomes observed in Hoechst 33258-treated L-cells

Treatment time	No. of laggards1 no. of cells studied
35 μg/ml Hoechst 33258	
30 min	6/150
1 h	25/160
3.5 h	64/150
74 h	721150
Control	31150

Determination of laggards. Routine chromosome preparations made by using spindle arrestants are generally unsuitable for critical evaluation of the existence of laggards. We therefore resorted to Giemsa-stained chromosome preparations without the use of Colcemid or hypotonic. We also carried out double staining, differential staining of spindle and chromosomes, as defined above. Using these techniques the laggards could be unambiguously identified.

When cells were analysed after double staining, a direct relationship between the number of laggards and duration of exposure of cells to Hoechst 33258 was observed. The data presented in Table I show that up to 30 min exposure to 35 μg/ml of Hoechst 33258 does not produce laggards more often than is observed in the control. However, a 60-min exposure increased the frequency by 6-fold over the control value. Longer exposures produce proportionately more aberrations. Apparently, chromosomes of all sizes were excluded from the spindle as laggards. In **a** few

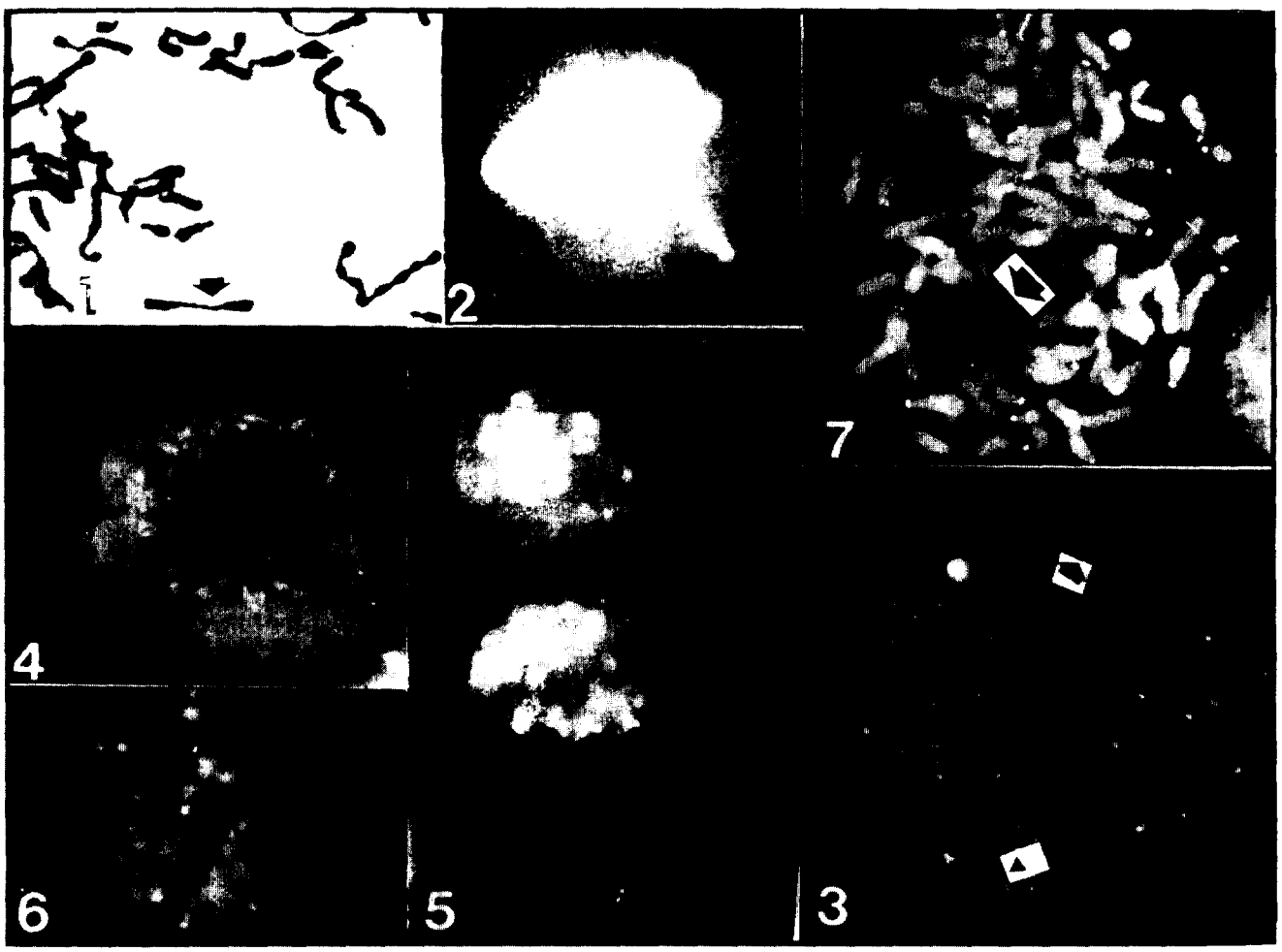

Fig. 1. A Hoechst 33258-treated cell showing decondensation of pencentric heterochromatin especially noticeable in two-armed chromosomes. **Fig. 2.** A cell processed by double staining technique (discussed in the text) showing the octacentric chromosome lying outside the spindle zone. **Fig. 3.** Antikinetochore antibody staining of an L-cell showing only one kinetochore site per chromosome including the octacentric (large arrow). Also note a minute chromosome composed almost entirely of a kinetochore. **Fig. 4.** Polar view of a cell showing antikinetochore antibody stained kinetochores in a ring. **Fig. 5.** A Hoechst 33258-treated cell similar to that in Figure **4.** A chromosome with clearly visible kinetochore dots is lagging. Also note micronuclei without kinetochore dots. **Fig. 6.** A centric fragment or a complete chromosome-carrying micronucleus showing kinetochore dots. **Fig. 7.** Antikinetochore antibody-treated L-cell showing kinetochores in the region of decondensed pericentric heterochromatin. Also observe two types **of** acrocentrics: those with a very short arm and those without any apparent short arm.

instances the longest chromosome (the octacentric marker) was also observed to lie outside the spindle zone (Figure 2). Superficially, it appears that all or most of these laggards represent a whole chromosome.

Hoechst 33258 is known to induce chromosome aberrations, preferentially in A:T-rich heterochromatin. So it is likely that some of the laggards arise from chromosomes which had lost the centromeric region through breakage. Hoechst 33258-treated cells showed bridges and fragments at anaphase testifying to the occurrence of chromosome breakage. In addition, 51/730 anaphase cells in the 35 μg/ml Hoechst 33258-treated population expressed micronuclei. To distinguish between whole chromosome laggards and large acentric fragments we applied the antikinetochore antibody to these cells which should distinguish between the two types of laggards and micronuclei, i.e. those with and without kinetochores.

Application of antikinetochore antibody. A routine preparation of L-cells to show kinetochore proteins displays no noteworthy differences between the various types of chromosomes. Apparently, all chromosomes, whether dicentrics originating from Robertsonian translocations or multicentrics like the previously alluded to octacentric, show only one pair of kinetochore dots (Figure 3). The so-called acrocentrics, with rare exceptions, appear to have terminal kinetochores. This may be due to the truly terminal location of the kinetochore proteins or due to an undetectably small short arm.

When Hoechst 33258-treated cells were prepared without application of Colcemid or hypotonic, the chromosomes appeared to follow the expected plane of orientation around the periphery of the spindle (polar view in Figure 4) and the laggards could be readily identified (Figure 5). However, there were occasional anaphase cells which showed kinetochore dots 'stuck' at or near the equatorial region after other chromosomes had migrated to the poles. This may indicate that exchanges between two chromosomes (or a U-shaped intrachromosal exchange) occurred in the vicinity of the centromere(s). Several cells had chromatid bridges without any evidence of a kinetochore being present in the equatorial region of the spindle.

The fate of laggards was obvious in cells at telophase. Antikinetochore antibody staining showed micronuclei without (Figure 5) or with (Figure **6)** kinetochores, isolated chromosomes which had not yet formed micronuclei (Figure 5) and acentric fragments.

It has been reported that pericentric heterochromatin when decondensed with Hoechst 33258 may not form a kinetochore (Lica *et al.*, 1982). In our preparations we did not observe any such aberration; all chromosomes, even with extensive decondensation, responded to antikinetochore antibody label (Figure 7). In some cells which had two copies of the marker octacentric, each showed one kinetochore. In spite of the decondensation of heterochromatin the chromosomes appeared to undergo normal centromere separation (Vig and Swearngin, 1985) and the size of kinetochore dots located in such extended heterochromatic regions was not affected. Thus, it appears that decondensation of pericentric heterochromatin has no effect on the morphology of the kinetochore or its basic function. Such cells do traverse through mitosis. However, if a cell had undergone chromosome breakage, many fragments showed a lack of kinetochore formation. These, evidently, represented acentric fragments. None the less, some chromosomes were exclusively made up of a small pair of kinetochore dots (Figure 3) and no evidence of extended peripheral heterochromatin was available. Such microchromosomes were observed mostly in Hoechst 33258-treated cells and also, albeit rarely, in untreated cells. In routine Giemsa-stained cells such minute chromosomes could be ruled out as Giemsa-positive, non-chromosomal particles.

In summary, Hoechst 33258-treated chromosomes develop kinetochores of normal appearance and function. The formation of laggards and micronuclei seen in our preparations could possibly arise from the malformation of one or a few microtubules at the point of attachment to kinetochores. The existence of microchromosomes which can be best detected by use of antikinetochore antibody is also of interest.

Caffeine- and bleomycin-treated cells

Mouse L-cells and pig kidney cells were treated with 0.3% caffeine solution for a period of 2 h. After **12** h of recovery, a sample of the treated population was analysed for chromosome aberrations. The results from two experiments are given in Table II: 1 and 2. Clearly, the incidence of free fragments increased; however, the frequency of exchanges did not. The data support the previous reports (see Kihlman, 1977) that caffeine at 37°C does not permit rejoining of broken ends of chromosomes. When the same cell populations were allowed to recover for an additional period of 12 h, presumably into the next cell cycle, a large frequency of micronuclei was also recorded. Since these studies are aimed at elucidating neither the relationship between the frequency of micronuclei in the two succeeding generations nor between aberrations and micronuclei, no effort was made to establish a relationship between the two end results of the cytological damage induced by caffeine.

When the cell population used in the study of micronuclei was treated with antikinetochore antibody, two types of micronuclei were observed. One of these showed a lack of any antikinetochore antibody binding site and was, therefore, inferred to be made up of acentric fragments. The other type displayed kinetochores similar to those observed in interphase cells containing the main nucleus. The size of various micronuclei was not constant; some being several times larger than the smaller ones. The larger ones showed more than one, and sometimes as many as eight, kinetochore dots. These perhaps constituted an *en masse* elimination of several chromosomes from the main nucleus. However, most micronuclei contained between 0 and 3 kineto-

Table II. Type and frequency of Chromosome aberrations and micronuclei observed in caffeine- or bleomycin-treated cells

Treatment cell	Cells	Chromosome aberrations"				Micronuclei	
Caffeine (0.3%)		B'	B"	C'-C'	C"-C"	Kinet	Kinet$^+$
1[b] L-cells	100	**26**	28	4	–		
2 L-cells	400	164	116	1	–		
3 L-cells	1000					30	31
4 L-cells	1000					116	*31*
5 Pig kidney	600					25	**31**
6 Pig kidney	575					17	16
Bleomycin (0.001%)							
7 L-cells	100	16	–	42	2		
8 L-cells	1000					33	16
Control							
L-cells	100	2	1	–	–		
L-cells	1000					2	*1*
Pig kidney	100	1	3	–	–		
Pig kidney	1000					1	1

"**B'** = chromatid-. **B"** = chromosome-type breaks; C'-C' = chromatid. C"-C" = chromosome-type exchanges; Kinet$^-$ = without. Kinet$^+$ = with kinetochore **dots.**

[b]Refers to experiment number.

chore dots. The data from some experiments are reproduced in Table 11: 3 – 6.

Treatment of L-cells with bleomycin also resulted in chromosome-type aberrations (Table II). However, a significant number of these appeared as chromatid-type exchanges. The preliminary data of the analysis of micronuclei, recovered 36 h after 0.001% bleomycin treatment, showed 1 – 3 kinetochore dots. A few micronuclei also showed up to six kinetochore antibody binding sites. The average number of kinetochores per micronucleus in the bleomycin-treated population was <2 in contrast to the caffeine-treated cells which had ~ 2.5 such dots.

Clearly, these results are only preliminary and no definite conclusions of a quantitative nature can be drawn. However. the study demonstrates the usefulness of the antikinetochore antibody technique to see whether a given micronucleus has resulted from acentric chromosome fragment(s) or whether it carries a kinetochore.

Discussion

Our interest in the study of Hoechst 33258 was generated by two observations. Firstly, Lica *et al.* (1982) have reported a loss of an antikinetochore antibody binding site on centromeres of Hoechst 33258-treated chromosomes. Secondly, recent studies from our laboratory demonstrated non-disjunction of the marker chromosome when treated with Hoechst 33258. Our studies also showed that the pattern of centromere separation in Hoechst 33258-treated cells is modified. A strict correlation between increasing amount of pericentric heterochromatin (repetitive DNA) and delayed centromere separation is a characteristic of untreated cells (Vig and Zinkowsky, 1985). In Hoechst 33258-treated cells it is disturbed (Vig and Swearngin, 1985). We therefore wondered if these effects can be related to a lack of formation of kinetochore in normal chromosomes as is sometimes observed for the accessory centromeres in dicentric chromosomes (Eamshaw and Migeon, 1985). The results from these studies show that any disturbance in the sequence of centromere separation is not correlated to a lack of kinetochore formation in Hoechst 33258-treated cells showing decondensation of pericentric heterochromatin. The kinetochore formation appears normal and there is no decondensation of the kinetochore region accompanying this phenomenon in the pericentric heterochromatin. It is, however, entirely likely that the laggards — in spite of showing kinetochores of apparently normal appearance — lack a particular component of the kinetochore protein which is not detectable by the particular CREST serum used in our study. Earnshaw and Rothfield (1985) have shown that different sera bind to different components of kinetochore proteins. At least three different proteins (17, 80 and 140 kd) have been identified as making the kinetochore complex. Alternatively, Hoechst 33258 may affect spindle microtubules in such a way as to render them ineffective in binding to some kinetochores.

Mouse kinetochores show terminal location on the so-called acrocentrics. It is not known if these chromosomes have a truly terminal centromere or if there is small undetectable short arm in all chromosomes. In some chromosomes, however, a non-terminal kinetochore location can be readily visualized as seen in Figure 7. This point is of interest in that Chromosomes having originated through Robertsonian translocations show only one kinetochore. If two acrocentrics undergo a whole Chromosome translocation, one should observe two pairs of kinetochore dots next to each other. However, this is not the rule in our cell line. None the less, in some cells in which chromosome breakage was induced with the help of bleomycin, we did observe certain chromosomes with more than one pair of kinetochore dots, as has also been reported for some multicentric rat chromosomes (Zinkowski *et al.*, 1986).

Another point of interest is the observation of microchromosomes which appear to be made up of nothing more than a mere pair of kinetochore dots. Their undetectability in routine Giemsa-stained preparations cautions us about their possible significance in cell survival. These chromosomes, which were observed in certain cell lines, e.g. SEWA-Rec 4 (Levan *et al.*, 1981), have been taken as evidence of the existence of double minutes; but no HSRs have been seen in cells which lack any such chromosomes. In the absence of any data on antikinetochore antibody binding one is led to wonder if the double minutes observed in other cell lines are really devoid of kinetochores.

The studies carried out with caffeine and bleomycin, although preliminary, are presented here to show that micronuclei can now be readily classified into two categories. Those arising from acentric fragments do not contain antikinetochore antibody binding sites while those with centric fragments or whole chromosomes show kinetochore dots. The micronuclei showing more than one kinetochore in bleomycin-treated cells may originate from translocations involving two or more centromeres but no such conclusions can be drawn from caffeine-treated cells since it does not produce translocations (Kihlman, 1977). Clearly, further studies are needed with caffeine in this test system. This test system, however, appears to be overly sensitive in that micronuclei with multiple kinetochores were not uncommon in any of the treated cells. It may well be that most cell lines with an inherent instability will not prove to be useful indicators for aneuploidy testing. None the less, the fact that micronuclei can be classified as carrying acentric or centric fragments (or whole chromosomes) can be used with more suitable test systems

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