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Immunofluorescent staining of kinetochores in micronuclei: a new assay for the detection of aneuploidy

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Summary

The immunofluorescent staining of kinetochores in micronuclei with antikinetochore antibodies was used to develop an in vitro assay for aneuploidy-inducing agents. The results show that about 80% of micronuclei induced by either colchicine or chloral hydrate contained kinetochores; only 9% of X-ray-induced micronuclei reacted positively to the antibody.

These findings indicate that the in vitro micronucleus assay coupled with immunofluorescent staining of kinetochores can be a useful method for assessing the ability of chemicals to induce aneuploidy and/or chromosome aberrations.

The development of test methods for assessing the ability of chemicals to induce aneuploidy is a major goal in the field of genetic toxicology, due to the fact that many agents inducing aneuploidy do not interact with DNA and are not detected in conventional assays for mutagens.

Most of the presently available tests for detecting aneuploidy use submammalian species such as fungi or ***Drosophila melanogaster*** and genetic end points. Detection of aneuploidy in mammalian cells is based primarily on cytogenetic assays such as metaphase chromosome counting or analyses of lagging chromosomes in anaphase, multipolar spindles or C-mitotic effects. None of these methods, however, has been sufficiently validated and there is a need for development, standardization and validation of assay procedures (Dellarco et al., 1986; Oshimura and Barret, 1986).

Micronuclei originate either from acentric chromosome fragments or from whole chromosomes/chromatids that are not incorporated into daughter nuclei at the time of cell division. The micronucleus assay can therefore potentially detect both clastogens and agents that affect the regular distribution of chromosomes at mitosis. However, the assay cannot characterize the nature of the nuclear disturbance induced by a tested compound (Schmid, 1975).

Many attempts have been made to discriminate between micronuclei deriving from chromosome

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breakage and those deriving from spindle malfunction by measuring either the size (Yamamoto and Kikuchi, **1980;** Hogstedt and Karlsson, **1985;** Wakata and Sasaki, **1987)** or the **DNA** content of micronuclei induced with different agents (Heddle and Carrano, **1977;** Nusse and Kramer, **1984;** Pincu et al., **1985).**

The recent discovery that sera from scleroderma patients (CREST variant) contain antikinetochore antibodies (Moroy et al., **1980)** has made it possible to visualize kinetochores both in metaphase and in interphase cells by indirect immunofluorescence staining (Brenner et al., **1981;** Earnshaw and Rothfield, **1985;** Hadlaczky et al., 1986).

In this study the immunofluorescent staining of kinetochores in micronuclei using a **CREST** serum has been used to develop an in vitro assay for aneuploidy-inducing agents. Micronuclei and chromosomal aberrations were studied in a Chinese hamster cell line after treatment with X-rays, colchicine or chloral hydrate, a metabolite of trichloroethylene known to induce mitotic nondisjunction in *Aspergillus nidulans* (Crebelli et al., **1985)** and aneuploidy in secondary spermatocytes of mice (Russo et al., **1984).**

Immunofluorescence studies using a CREST serum were performed to determine the frequency of kinetochore-containing micronuclei.

Materials and methods

Cell line and treatments

The hamster cell line Cl-1 was kindly provided by Prof. B.A. Kihlman, University of Uppsala. The cell line is a clonal derivative of male embryonic lung cells with a modal number of **22** chromosomes **(85%** of the cells) and a relative frequency of 1% polyploid cells. Cells were routinely grown in Ham's F-10 medium supplemented with 10% fetal calf serum and antibiotics at **37°C** in a humidified atmosphere containing 5% CO, in *air.* Under these conditions the average cell cycle length is about **18** h.

One day before the treatments cells were seeded onto coverslips in **40-mm** petri dishes. Cultures were X-irradiated **using** a Gilardoni apparatus **(180** kV, **6 mA,** 3 mm Al) or treated with colchicine (Merck) or chloral hydrate (Fluka A.G.) 'according to the experimental schedule in Fig. **1.**

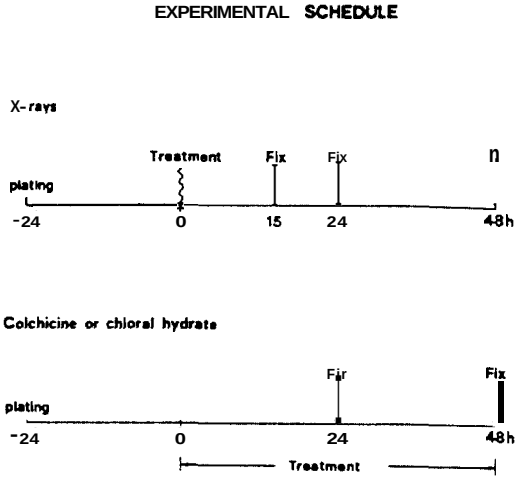


Fig. 1. Experimental schedule. For **each sampling time** 5×10^{-5} **M colchicine was added to half the samples** 3 h **before fixation to score for chromosomal aberrations.**

Preparation of slides and scoring for micronuclei and chromosomal aberrations

Micronuclei were scored in cells fixed after a short hypotonic treatment (3 min in 0.075 M KCl) according to Danford **(1984),** to preserve the cytoplasm around the nucleus. Chromosomal aberrations were observed in colchicine-arrested cells after a standard hypotonic treatment. A diameter of 50% of late interphase nuclei was chosen as the borderline between micronuclei and small nuclei. For each experimental point the frequencies of micronuclei were determined in 1000 cells and 100 metaphases were scored in colchicine-arrested cells for chromosomal aberrations.

Indirect immunofluorescence

Human serum LU851 containing antikinetochore autoantibodies was a generous gift of Dr. N.R. Ringertz (Karolinska Institut, Stockholm). The serum was obtained from a patient with the form of progressive systemic sclerosis known as CREST syndrome. The specificity of the antiserum is described in Hadlaczky et al. **(1986).**

Cells seeded on coverslips were either X-irradiated, or received colchicine or chloral hydrate (see Fig. **1).24** h later coverslips were rinsed 3 times in **Hank's** balanced salt solution and fixed in methanol for 30 min on ice. After rinsing **5** times in ice-cold phosphate-buffered saline (PBS), the

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Results

Table 1 shows
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TABLE 1

CELLS WITH MIC
COLCHICINE OR C

1000 cells were scored

Treatment	
Control	
X-Rays (Gy)	
0.5	
1	
2	
Colchicine ($M \times 10^{-9}$	
1	
10	
50	
100	
Chloral hydrate (M x	
1	
5	
10	
15	

Colchicine or chloral
experiments.

preparations were treated overnight at 4°C with 1:500 diluted antikinetochore serum in PBS–BSA (PBS containing 1% bovine serum albumin). The preparations were washed 5 times for 5 min in PBS–BSA and then exposed for 45 min at 37°C to rhodamine-conjugated rabbit anti-human IgGs diluted 1:1000 in PBS–BSA. After extensive washing in PBS–BSA and PBS, coverslips were stained for 10 min in 0.5 μg/ml Hoechst 33258 and mounted on slides in a PBS–glycerol (1:1) mixture. The preparations were examined with an Olympus Vanox-T microscope with AH2-RFA fluorescence equipment under either ultraviolet (for Hoechst) or green (for rhodamine) illumination.

Results

Table 1 shows a dose-dependent increase in the frequency of micronuclei in X-irradiated Cl-1 cells and a decline in the frequency of micronuclei with time. Within the tested dose range, colchicine or chloral hydrate induced micronuclei when given as continuous treatments for 24 or 48 h, the latter treatment being more effective. Higher doses of neither chemicals could be tested for micronucleus induction, since a strong C-mitotic effect was observed (data not shown). In Table 2 the frequencies of chromosomal aberrations after X-rays, colchicine or chloral hydrate are shown. As expected, X-rays were found to be very effective in inducing chromosomal aberrations while no significant increase in the frequencies of chromosomal aberrations was observed after colchicine or chloral hydrate.

To distinguish micronuclei due to chromosome breakage from those due to chromosome loss following treatments with mutagens with different mode of action, the immunofluorescent staining of the kinetochores was applied to X-ray, colchicine or chloral hydrate treated cells.

Cells were stained with the DNA-specific dye

TABLE 1

CELLS WITH MICRONUCLEI (I) AND NUMBER OF MICRONUCLEI (II) PER 1000 CELLS INDUCED BY X-RAYS, COLCHICINE OR CHLORAL HYDRATE IN Cl-1 CELLS

1000 cells were scored for each treatment.

Treatment	Fixation time following the treatments (h)					
	15		24		48	
	I	II	I	II	I	II
Control	4	4	3	3	4	4
X-Rays (Gy)						
0.5	59	67	49	53	41	44
1	136	159	117	134	70	103
2	192	232	179	213	112	122
Colchicine ($M \times 10^{-9}$)						
1	–	–	11	11	11	14
10	–	–	12	13	15	17
50	–	–	16	16	14	14
100	–	–	19	20	25	25
Chloral hydrate ($M \times 10^{-4}$)						
1	–	–	5	5	7	7
5	–	–	15	16	12	12
10	–	–	21	22	35	37
15	–	–	33	36	49	51

Colchicine or chloral hydrate were given as continuous treatments. The results in the table were consistently reproduced in 3 separate experiments.

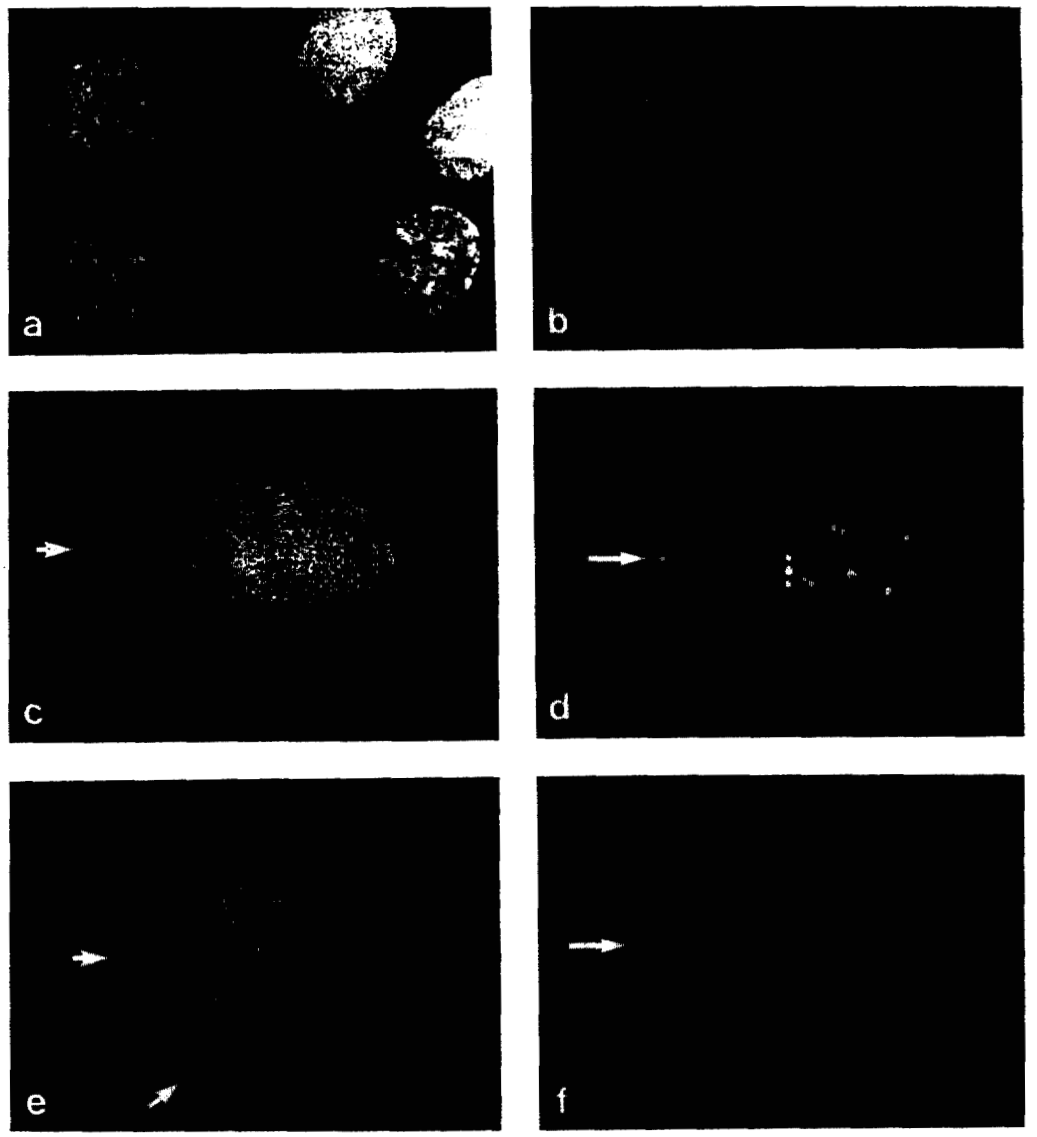


Fig. 2. Immunofluorescent staining of Cl-1 cells. The left panels depict Cl-1 cells stained with the DNA-specific dye Hoechst 33258. In the right panels, the same cells are stained with the antikinetochore antibody: (a,b) untreated cells; (c–f) examples of cells with micronuclei (short *arrows)* in colchicine-treated cultures; long arrows point to kinetochores in micronuclei stained using the antikinetochore antibody. In Fig. 2f only one micronucleus is positive to the antikinetochore antibody.

Hoechst 33258 to recognize the micronuclei, which were then analyzed for the antibody staining (see 'Fig. 2).

Table 3 shows that 46% of micronuclei in untreated cultures contained kinetochores; more than 80% of micronuclei induced by colchicine or chlo-

TABLE 2

CHROMOSOMAL A

100 cells were scored f

Treatment
Control
X-Rays (Gy)
0.5
1
2
0.5
1
2
Colchicine ($M \times 10^{-9}$
10
50
100
Chloral hydrate (M x
1
5
10
15

Colchicine and chlora
* Significant accordii
The results in the tab

TABLE3

PERCENTAGE OF

Treatment
Control
X-Rays (Gy)
0.5
1
Colchicine ($M \times 10^{-5}$
50
100
Chloral hydrate (Mx
10
15

Cultures were proces
obtained in 3 separa

TABLE 2

CHROMOSOMAL ABERRATIONS INDUCED BY X-RAYS, COLCHICINE AND CHLORAL HYDRATE IN Cl-1 CELLS

100 cells were scored for each treatment.

Treatment	Fixation time (h)	Abnormal cells (%)	Aberrations per 100 cells					
			Gaps	Chromatid breaks	Isochromatid breaks	Chromatid exchanges	Dicentrics + rings	Total (excluding gaps)
Control	24	6'	3	2	1	0	0	3
X-Rays (Gy)								
0.5	15	8	2	0	3	0	3	6
1	15	19	0	7	11	2	0	20 •
2	15	40	6	5	50	4	10	69 *
0.5	24	8	2	3	3	0	0	6
1	24	20	2	1	7	0	11	19 •
2	24	38	5	15	20	0	14	49 *
Colchicine ($M \times 10^{-9}$)								
10	24	10	9	5	0	0	0	5
50	24	9	8	0	5	3	0	8
100	24	8	5	0	3	0	0	3
Chloral hydrate ($M \times 10^{-4}$)								
1	24	8	7	2	0	0	0	2
5	24	13	9	7	0	0	0	7
10	24	12	12	3	1	0	1	5
15	24	8	7	1	2	0	1	4

Colchicine and chloral hydrate were given as continuous treatments.

• Significant according to Student's t-test: $p < 0.05$.

The results in the table were consistently reproduced in 3 separate experiments.

TABLE 3

PERCENTAGE OF MICRONUCLEI WITH KINETOCHORES (CREST-POSITIVE)

Treatment	Number of analyzed cells	Number of micronuclei	Micronuclei per 1000 cells	% Micronuclei with kinetochores (CREST-positive)
Control	9000	79	8.7	46.8
X-Rays (Gy)				
0.5	1500	66	44.0	9.1
1	1000	146	146.0	8.9
Colchicine ($M \times 10^{-9}$)				
50	1500	26	17.3	84.6
100	5000	106	21.2	83.9
Chloral hydrate ($M \times 10^{-4}$)				
10	4000	69	17.2	79.7
15	3000	97	32.3	89.3

Cultures were processed for immunofluorescent staining 24 h after the beginning of the treatments. The table summarizes the results obtained in 3 separate experiments.

Hoechst 33258.
es of cells with
ined using the

uclei in un-
; more than
ine or chlo-

ral hydrate reacted positively to the antibody staining but only 9% were positive after X-rays.

Discussion

Our results show that the in vitro micronucleus assay together with the immunofluorescent staining of the kinetochores can be used to screen chemicals for their ability to induce mitotic aneuploidy. In this assay the observed end-point, namely micronuclei containing whole chromosomes, may be taken as evidence for a potential induction of aneuploidy (hypoploidy).

Our data show that the micronuclei induced by X-rays are likely to originate from chromosome fragments lagging at anaphase while the micronuclei induced by colchicine result from lagging of whole chromosomes. In fact, no significant induction of chromosomal aberrations is observed with this chemical (Table 2). Furthermore, our data indicate that chloral hydrate is a mitotic poison in mammalian cells in vitro since it is shown to induce micronuclei without inducing chromosomal aberrations (Table 2), in accordance with previous results obtained in mammalian male germ cells (Russo et al., 1984). The increase in the frequencies of micronuclei in cells fixed at 48 h after the beginning of treatment with colchicine or chloral hydrate (see Table 1) indicates that an experimental design with continuous treatments and sample times of approximately 2 cell cycles improves the detection of aneuploidy-inducing agents in in vitro assays (Galloway and Ivett, 1986). A further improvement in the detection of micronuclei is the use of a mild hypotonic treatment, as already pointed out by Hogstedt (1984) and Danford (1984).

However, the direct evidence for a micronucleus containing a whole chromosome is only obtained using techniques for identifying centromeres or associate materials. Previous work by Banduhn and Obe (1985) showed that 50% or more of the micronuclei induced by colchicine in human lymphocytes in vitro contained chromocentres as detected by C-banding; with the same technique only 15% of X-ray-induced micronuclei could be shown to contain centromeres (Obe and Beek, 1975).

The immunofluorescent staining of the kinetochores by the CREST antibody greatly improves the detection of centromere-associated material in micronuclei. In fact, in our experiments about 80% of the micronuclei induced by colchicine or chloral hydrate reacted positively to the antibody (Table 3). In untreated cells about 50% of the micronuclei were CREST negative (Table 3), indicating that both spontaneous chromosomal aberrations and numerical changes are involved in the spontaneous production of micronuclei in this cell line.

The presence of CREST-negative micronuclei in untreated cultures could account for the micronuclei negative to the staining we observed in colchicine or chloral hydrate treated cells. On the other hand, the incidence of CREST-positive micronuclei at the highest dose of X-rays could indicate an induction of aneuploidy by this agent. X-irradiation has already been reported to induce aneuploidy in Drosophila and mammalian germ cells in vivo (Bond and Chandley, 1983; Tease, 1982; Russo et al., 1983).

In conclusion, our data demonstrate that the in vitro micronucleus assay can be used to screen for aneuploidy-inducing agents, when coupled with the antikinetochore staining.

A relevant feature of this assay is that the determination of the origin(s) of micronuclei by the immunofluorescent staining of kinetochores provides a direct information on the relative effectiveness of a given chemical in inducing different genetic damages (chromosomal aberrations vs. numerical changes). Further developments of the assay will be accomplished by using primary cell cultures with low levels of spontaneous aneuploidy (Galloway and Ivett, 1986) and culturing the cells in the presence of 5-bromodeoxyuridine to control the proliferation kinetics of treated populations (Heddle et al., 1981; Lasne et al., 1984).

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