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# Kinetochore identification in micronuclei in mouse bone-marrow erythrocytes: An assay for the detection of aneuploidy-inducing agents

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## Summary

An in vivo micronucleus assay using mouse bone marrow for identifying the ability of chemicals to induce aneuploidy and/or chromosome breaks is described. Micronucleus formation in bone-marrow erythrocytes of mice is commonly used as an index for evaluating the clastogenicity of environmental agents. However, micronuclei may also originate from intact lagging chromosomes resulting from the effect of aneuploidy-inducing agents. We have used immunofluorescent staining using anti-kinetochore antibodies to classify micronuclei for the presence or absence of kinetochores. Micronuclei positive for kinetochores are assumed to contain intact chromosomes and result from induced aneuploidy; while those negative for kinetochores contain acentric chromosomal fragments and originate from clastogenic events. The assay was evaluated using X-irradiation (a known clastogen) and vincristine sulfate (an aneuploidy-inducing agent). A dose-related response for the induction of micronuclei was observed for both agents. Micronuclei induced by X-irradiation were negative for kinetochores while the majority of the micronuclei resulting from vincristine treatment contained kinetochores. Thus, the micronucleus assay in combination with immunofluorescent staining for kinetochores may provide a useful method to simultaneously assess the ability of chemicals to induce aneuploidy and/or chromosome breaks.

Aneuploidy, while a predominant cause of birth defects in the human population, may also be involved in the development of neoplasia (Oshimura et al., 1988; Conti et al., 1986; Cavenee. 1983). Recent studies have shown that environmental chemicals may play a significant role in the etiology of aneuploidy (Barrett et al., 1987; Dellarco et al., 1986; Oshimura and Barrett, 1986). Several in vitro bioassays using rodent and human cells to identify potential aneuploidy-inducing agents have recently been developed (Sandhu et al., 1988; Degrassi and Tanzarella, 1988; Thomas and Parry, 1988; Eastmond and Tucker, 1989). In this article we describe an in vivo assay in which micronuclei are examined for the presence of kinetochores using anti-kinetochore antibodies to distinguish between clastogenic and/or aneuploidy inducing agents.

Micronuclei formation in polychromatic erythrocytes (PCE) is a commonly used assay for the identification of clastogenic agents (Heddle et al.,

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1983; Schmid, 1975; Heddle, 1973). However, micronucleation in PCE may result from failure of either an intact chromosome or an acentric chromosomal fragment to incorporate into the main nucleus at the preceding anaphase. Therefore. a classification of micronuclei for the presence of a centric or acentric chromosome would provide a method to distinguish between aneuploidy inducing and clastogenic agents. Attempts for such an analysis have been made on the basis of the size of the micronuclei (Wakata and Sasaki, 1987; Hogstedt and Karlsson. 1985; Yamamoto and Kikuchi, 1980); DNA content (Heddle and Carrano, 1977) and presence of centric heterochromatin in micronuclei (Banduhn and Obe, 1985). Each of these methods in addition to being laborious, suffers from ambiguities. Another approach used recently is based upon qualitative analysis for the presence of kinetochores in micronuclei induced in fibroblasts (Degrassi and Tanzarella, 1988; Henning et al., 1988; Thomas and Perry, 1988) and lymphocytes (Eastmond and Tucker, 1989).

Antibodies present in serum of patients with the autoimmune disease scleroderma CREST syndrome (Moroi et al., 1980; Fritzler et al., 1980) specifically bind to the kinetochores in metaphase chromosomes as well as in interphase nuclei of human and other species (Brenner et al., 1981; Brinkley et al., 1985). These antibodies therefore provide a useful probe for the detection of kinetochores in micronuclei. In the present study we have used CREST antibodies for staining to detect kinetochores in micronuclei in bone marrow erythrocytes of mice exposed to ionizing radiation and vincristine sulfate. Kinetochore proteins are bound to centromeric DNA in chromosomes; the procedure therefore has the capability of distinguishing the micronuclei containing centric chromosomes from those containing acentric fragments, and therefore indicating the probable mechanism of micronuclei formation.

## Materials and methods

### *Animals and chemical treatment*

Random bred, SW male mice weighing about 20 g (8–10 weeks old) were used in these experiments. Vincristine sulfate (VCR) (Sigma, St. Louis, MO) and X-irradiation, known to induce aneuploidy and chromosome breaks, respectively, were used as test agents. VCR was administered by interperitoneal injection at doses of 0.10, 0.20 0.50 and 1.0 mg/kg body weight; while X-rays were given as whole-body irradiation at doses of 100, 150, 200 and 250 rad using a Phillips RT200 X-ray machine. Two mice per dose were treated with VCR and X-ray and two animals were maintained as controls.

### *Preparation of smears*

Animals were sacrificed 24 h after treatment by cervical dislocation. Both femurs were removed by dissection and bones were cleaned of adhering muscle tissue. Marrow was flushed out into a 15-ml centrifuge tube using a hypodermal syringe containing 1 ml fetal bovine serum equilibrated to 25 mM EDTA (FBS). A homogeneous suspension of bone-marrow cells was prepared in FBS by gentle mixing with a Pasteur pipette. Cells were then centrifuged to pellet, resuspended gently in a few drops of FBS, transferred onto a clean microscope slide and smeared with the edge of a coverslip as described (Schmid, 1975).

Bone-marrow smears were allowed to dry for 20–30 sec and then fixed with cold (−20°C) absolute ethanol. Following 30 min fixation, slides were warmed to room temperature, sequentially hydrated in 80%, 50%, 30% and 10% ethanol diluted with Dulbecco's phosphate-buffered saline (PBS), and then transferred into PBS. The sequential hydration in decreasing concentration of ethanol is necessary to avoid the loss of cells from smears which could result if transferred directly to PBS from absolute ethanol. Fixed smears were either stained immediately or stored in PBS for a week.

Metaphase spreads for kinetochore staining were prepared from mouse fibroblast cell line RAG. Cells arrested in mitosis with colcemid (0.2 μg/ml) for 2 h were harvested by mitotic shake off (Athwal and Sandhu, 1985). After a brief treatment with hypotonic solution (0.075 M KCl) cells were deposited on microscope slides using a cytocentrifuge and fixed in cold (−20°C) absolute ethyl alcohol. Fixed slides were either used immediately or stored at 4°C for later use.

### *Staining for kinetochores and scoring*

The serum from a scleroderma CREST syn-

drome patient containing antikinetochore antibodies was kindly provided by Dr. **B.R.** Brinkley (Dept. of Cell Biology and Anatomy, University of Alabama, Birmingham). The FITC conjugated rabbit anti-human and goat anti-rabbit antibodies were purchased from Boehringer Mannheim. For staining, bone marrow smears were overlaid with 50 $\mu$l of CREST antiserum (diluted 1:500) covered with a coverslip and then incubated at 37°C in a humidified atmosphere. After **45** min, coverslips were removed and the slides were rinsed 3 times with PBS, each for 5 min, to remove unbound antibodies. Slides were then overlaid with 50 $\mu$l of FITC conjugated goat anti-human IgG (diluted 1:20), cover slipped and incubated again at 37°C in humidified atmosphere for **45** min. Antiserum was removed by rinsing the slides with PBS three times. Immunofluorescence staining of kinetochores was amplified by using FITC conjugated rabbit-anti-goat IgG (diluted 1:20) as a third antibody for staining. Slides stained with CREST antibodies were then stained with propidium iodide (1 $\mu$g/ml) for 30 sec. Excess stain was rinsed off with PBS, and slides were mounted in glycerol mixed with phenylene diamine 9:1 (Johnson and Nogueira-Araujo, 1981). Double staining of cells using propidium iodide for DNA and CREST antibodies for kinetochores makes it possible to simultaneously score erythrocytes for the presence of micronuclei and classify micronuclei for the presence or absence of kinetochores.

Stained slides were scored for the number of erythrocytes with and without micronuclei using an epifluorescence equipped Olympus microscope at an excitation and barrier filter combination of 400 nm and 490 nm, respectively. Micronucleated erythrocytes were further classified for the presence or absence of kinetochore staining. At least 2000 erythrocytes were scored for micronuclei from each mouse exposed to vincristine sulfate, X-rays and controls. Photomicrographs were prepared using Kodak ectachrome film EL135.

## Results and discussion

A schematic outline of the process of micronuclei formation in PCE is given in **Fig. 1.** Erythroblasts in the bone marrow undergo last chromosome replication and mitosis and differentiate to form polychromatic erythrocytes. Chromosomal breaks or interference in the mitotic process that result in lagging chromosomal material during this division lead to the formation of micronuclei in addition to the main nucleus in polychromatic erythrocytes. During differentiation, for yet unknown reasons, only the main nucleus is expelled from erythrocytes, leaving behind any micronucleus formed in erythroblasts. The identification of micronuclei in PCE by Giemsa staining (Heddle, 1973; Schmid, 1975; Heddle et al., 1983) does not distinguish micronuclei containing acentric chromosomal fragments from those with intact chromosomes. The double staining method, using anti-kinetochore antibodies and propidium iodide provides a method to simultaneously determine the frequency of micronucleated erythrocytes and distinguish between ones originating from acentric chromosome fragments and centric chromosomes (Degrassi and Tanzarella. 1988; Henning et al., 1988; Thomas and Perry, 1988; Eastmond and Tucker, 1989).

Immunofluorescent staining of kinetochore in metaphase chromosomes, interphase nuclei of mouse cells and micronuclei in bone-marrow erythrocytes is demonstrated in Fig. **2.** The yellow green kinetochore spots stained with CREST antibodies are distinctively identifiable in reddish background of propidium iodide-stained metaphase spreads as well as in interphase nuclei (Fig.

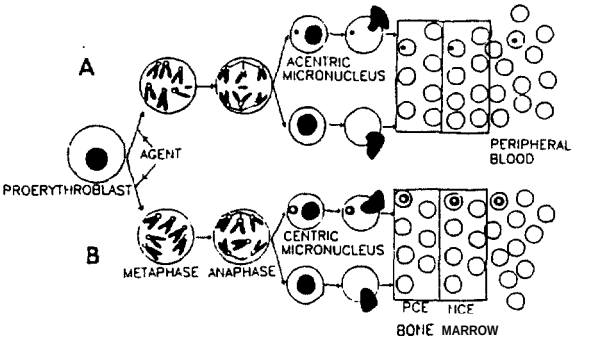


**Fig.** 1. Schematic diagram of the mechanism of micronuclei formation in bone-marrow erythrocytes resulting from acentric chromosomal fragments **and** intact chromosomes. Top panel: represents the formation of micronuclei due to chromosome breaks by **a** clastogen; bottom panel: represents micronuclei resulting from **an** intact lagging chromosome. Presence or absence of **a** kinetochore or centromere can be used to distinguish between two mechanisms of micronucleation.

2). In interphase nuclei, the number of kinetochores correspond to the genomic chromosome number of mice (Fig. 2). However a variable number of kinetochore spots were observed in different nuclei among the nuclei scored for kinetochore spots. This variation may result from overlap of individual spots in a nucleus, difference in the plane of focus of the microscope lens, and/or differential penetration of antibodies while staining (Eastmond and Tucker, 1989). The variation may also occur due to loss of antigenicity in individual kinetochores as a result of fixation. Several different fixation protocols involving the use of methanol: acetic acid and paraformaldehyde were tried, but adequate kinetochore staining was observed only when cold (−20°C) absolute ethanol was used.

The method of kinetochore staining to distinguish between clastogenic and aneuploidy-inducing effects was evaluated using X-rays and vincristine sulfate. Data on the kinetochore positive micronuclei induced by X-irradiation and VCR are presented in Table 1. Staining with propidium iodide does not distinguish between PCE and normochromatic erythrocytes (NCE). Therefore, the data presented in Table 1 are based on

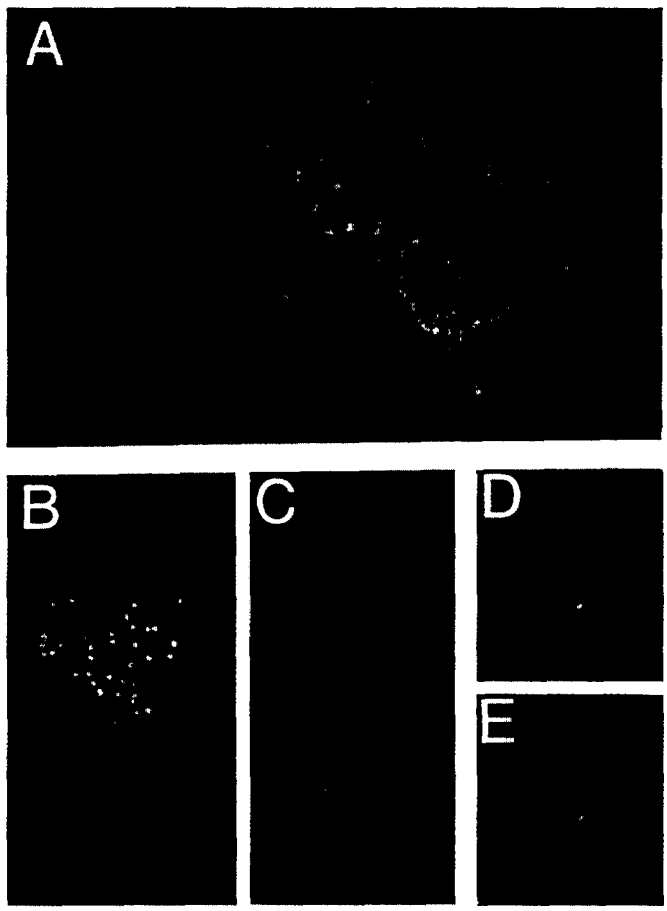


Fig. 2. Immunofluorescent staining with CREST antibodies for kinetochores and propidium iodide staining for DNA. (A) Mouse metaphase chromosomes stained with CREST anti-kinetochore antibodies showing two kinetochore spots on every chromosome. (B) Kinetochore staining in an interphase nucleus showing 40 spots corresponding to genomic chromosome number of mouse. (C), (D) and (E): Erythrocytes stained with CREST-antibodies and propidium iodide. In Panel C: two erythrocytes do not contain any DNA; one contains one micronucleus while the second contains,two micronuclei. A single micronucleus present in one of the erythrocytes negative for kinetochore staining; whereas one of the two micronuclei present in one of the erythrocytes is positive and the second is negative. Panel D: shows a single kinetochore positive micronucleus. Panel E represents one erythrocyte with two micronuclei both positive for kinetochore staining.

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TABLE 1

NUMBER OF MICRONUCLEATED ERYTHROCYTES AND MICRONUCLEI WITH KINETOCHORES PER 2000 ERYTHROCYTES

The numbers given represent an average for two animals for each dose of treatment.

| Dose | Number of micronucleated erythrocytes | Number of micronuclei with kinetochore spots |
|---|---|---|
| Control | 2 | 0 |
| X-Irradiation (rad) | | |
| 100 | 31 | 1 |
| 150 | 57 | 1 |
| 200 | 75 | 2 |
| 250 | 89 | 3 |
| Vincristine sulphate (mg/kg bw) | | |
| 0.10 | 63 | 56 |
| 0.20 | 124 | 98 |
| 0.50 | 65 | 31 |
| 1.00 | 30 | 26 |

the total number of erythrocytes scored. In X-irradiated mice the number of micronucleated erythrocytes increased with the dose and varied from 31(1.50%) at a dose of 100 rad to 89(4.45%) observed for a dose of 250 rad (Table 1). Among X-ray-induced micronuclei, only a small number (1–3) were positive for kinetochores at each dose level (Table 1).These data are consistent with the fact that X-rays predominantly induce chromosome breaks; the loss of an intact chromosome occurs with a very low frequency (Heddle and Carrano, 1977; Eastmond and Tucker, 1989).

In mice treated with VCR, the number of micronuclei increased from 63(3.10%) at a dose of 0.10 mg/kg body weight to 124(6.20%) at a dose of 0.20 mg/kg body weight. However, number of micronucleated erythrocytes 65(3.25%) and 30(1.50%) observed at doses of 0.50 and 1.00 mg/kg body weight respectively were lower than those observed at a dose of 0.20 mg/kg body weight (Table 1). A great majority (57–88%) of VCR induced-micronuclei showed the presence of bright kinetochores at all doses (Table 1, Fig. 2). VCR is known to induce aneuploidy, thus micronuclei in the erythrocytes of mice exposed to VCR are most likely to originate due to failure of an intact chromosome to incorporate into the main nucleus (Eastmond and Tucker, 1989). Lack of kinetochores in a low percent of the micronuclei was not surprising. Mitotic poisons have been reported to induce very low frequencies of chromosome breaks (Satya-Parkash et al., 1986). Therefore a low percentage of the micronuclei would be expected to lack kinetochore-specific fluorescence. In some cases lack of lunetochore staining may also result due to the loss of epitope or accessibility of the kinetochore protein resulting from fixation procedures.

The reduced frequency of micronuclei observed at increased concentrations (i.e. 0.50 and 100 mg/kg bw) of vincristine may be due to saturation of all target sites on tubulin. This would inhibit assembly of a functional spindle and arrest cells in mitosis. Release from the mitotic block will lead to the production of polyploid cells, rather than the loss or gain of individual chromosomes. Extrusion of a polyploid nucleus from an erythroblast will result in a normal erythrocyte. In comparison, a lower concentration of a mitotic poison will partially inhibit the spindle, resulting in nondisjunction of individual chromosomes rather than complete arrest of cells in mitosis. Thus a mitotic inhibitor would show a dose-related increase in the frequency of micronucleated 'erythrocytes up to an optimal concentration. Similar results were also obtained in our previous studies on cultured cells (Athwal and Sandhu, 1985; Sandhu et al., 1988). where increased frequencies of polyploid cells were observed with increased concentration of mitotic inhibitors. In addition, increased frequencies of polyploid cells at higher doses were accompanied by a corresponding decrease in the frequencies of aneuploid cells (Sandhu et al., 1988).

The fact that the majority of VCR-induced micronuclei are kinetochore-positive, while the ones induced by ionizing radiation are kinetochore-negative. shows the usefulness of the technique for detecting potential aneuploidy-inducing agents. The availability of a vast number of erythrocytes and the ease of identifying the presence of kinetochore-positive micronuclei make this assay particularly suitable for predicting the genetic hazard and mechanism of action of environmental agents.

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