Environmental and Molecular Mutagenesis 19:1–6 (1992)

Centromere Detection in Vinblastine- and Radiation-Induced Micronuclei of Cytokinesis-Blocked Mouse Cells by Using In Situ Hybridization With a Mouse Gamma (Major) Satellite DNA Probe

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Non-isotopic in situ hybridization using a mouse gamma (major) satellite probe DNA was applied to detect centromeres in micronuclei, which were induced in vitro in mouse liver cells by ionizing radiation and by vinblastine sulfate. In a cytokinesis-blocked micronucleus assay a dose-dependent induction of micronuclei was found for both agents. After vinblastine exposure the observed micronuclei showed centromere-positive hybridization signals in an order of magnitude of 70–90%, but after radiation exposure the magnitude was only 10–20%. Since the in situ hybridization technique detects centromeric DNA directly, it can be used in a cytokinesis-blocked micronucleus assay for a rapid and reliable discrimination between aneuploidy-inducing and clastogenic agents.

Key words: micronucleus assay, in situ hybridization, aneuploidy-inducing and clastogenic agents, centromere

INTRODUCTION

The cytokinesis-blocked (CB) micronucleus (MN) assay [Fenech and Morley, 1985] which allows for the influence of cell proliferation kinetics in MN expression provides an easy and rapid means for detecting chromosomal damage induced by chemicals or ionizing radiation. However, since MN may contain chromosomal fragments or whole chromosomes an MN assay must be able to distinguish between a clastogenic or an aneuploidy-inducing property of an agent. Several attempts have been made to overcome this problem by measuring either their sizes [Yamamoto and Kikuchi, 1980; Högstedt and Karlsson, 1985; Wakata and Sasaki, 1987] or their DNA content [Heddle and Carrano, 1977; Nüsse and Kramer, 1984; Pincu et al., 1985; Vanderkerken et al., 1989]. Evidence for the presence of whole chromosomes in MN came from the observation of chromocentres detected by C-banding [Banduhn and Obe, 1985], and from the re-expression of metaphase chromosomes when MN of human cells were fused with whole Chinese hamster cells [Viaggi et al., 1987]. Using anti-kinetochore antibodies from patients with the CREST variant of scleroderma [Moroi et al., 1980], the presence of kinetochores in MN was demonstrated by immunofluorescence staining in a series of reports [Brinkley et al., 1985; Frackowiak et al., 1986; Nüsse et al., 1987; Thomson and Perry, 1988; Degrassi and Tanzarella, 1988; Henning et al., 1988; Eastmond and Tucker, 1989a, b; Miller and Adler, 1990]. Recently, data on the use of centromere-specific DNA probes in non-isotopic in situ hybridization (ISH) for classification of MN have been presented. Becker et al. [1990] used the human alphoid p82H DNA probe [Mitchell et al., 1985] in a conventional (without cytochalasin B) MN assay with human lymphocytes. Miller et al. [1991] used a mouse gamma satellite DNA probe [Weier et al., 1991] in the mouse bone marrow polychromatic erythrocyte MN assay. We now show that ISH with this DNA probe is a suitable technique for detecting centromeres in induced MN of CB mouse cells in vitro.

MATERIALS AND METHODS

Cell Culture and Assay Procedure

The mouse liver cell line FMH-202 was used for the experiments. Cell culture conditions were performed as previously described [Salassidis et al., 1991]. Briefly, slide cultures were set up in Quadriperm dishes (Heraeus, Hanau, FRG). Cells were seeded at a density of 2×10^5 per dish and cultured in a medium containing 211 μg/ml arginine and 10% dialysed newborn calf serum (Gibco, Eggenstein,

Received April 12, 1991; revised and accepted July 15, 1991.

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FRG) to aid cell attachment. About 3 hr after seeding the medium was removed and replaced by serum-free MX-83 medium [Hoffman et al. 1989] containing insulin (10 μg/ml) and hydrocortisone (300 ng/ml).

After a further 21 hr cells were treated with the reference spindle poison vinblastine sulfate (VBL, Sigma, Deisenhofen, FRG) dissolved in phosphate-buffered saline (PBS) immediately before use. A dose of 0.08 μM was cytotoxic so 0.06 μM was used as the highest tolerated dose. Ionizing radiation was used as reference clastogen. Irradiation was performed with ^{137}Cs gamma-rays delivered at a dose rate of 1 $Gymin^{-1}$. After a 6 hr exposure to VBL, cells were washed with PBS and fed with fresh medium; 1 hr later, i.e., 31 hr after culture initiation, cytochalasin B (Cyt B, Aldrich, Steinheim, FRG) dissolved in dimethyl-sulfoxide (DMSO; final concentration 0.3% in the medium) was added to VBL- and radiation-exposed cultures at a final concentration of 3 μg/ml [Fenech and Morley, 1985] and left for a further 25 hr until harvesting. For cell preparation, the medium was removed and replaced by 5 ml of a pre-warmed hypotonic solution, consisting of a mixture of Hank's salt solution (2 volumes) and distilled water (1 volume) for 16 min at 37°C. The cells were then fixed 3 times with methanol:acetic acid (3:1), air-dried, and stored at −20°C under a nitrogen atmosphere until use.

In Situ DNA Hybridization (ISH)

A biotin-labeled degenerate DNA probe homologous to part of the 234 bp repeated mouse gamma (major) satellite DNA [Weier et al., 1991] was used. It was generated by primer-directed in vitro amplification of a gamma satellite probe DNA library by using the polymerase chain reaction (PCR) according to a procedure previously described by Weier and Rosette [1990]. ISH was carried out following the protocol of Pinkel et al. [1986, 1988] with slight modifications. Briefly, slides were pretreated in pepsin (100 μg/ml) at room temperature for 10 min, fixed in 4% paraformaldehyde in PBS plus 50 mM $MgCl_2$ for 10 min, and washed in 2 × SSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7.0). Biotinylated gamma satellite probe DNA (3 μl ca .60 ng) was mixed with 27 μl hybridization mix as described by Pinkel et al. [1988], so the final concentration was 55% formamide (FA), 10% dextran sulfate, 1 μg/μl sonicated herring sperm carrier DNA, 2 × SSC, pH 7.0. DNA in the hybridization mix was denatured at 72°C for 5 min, chilled on ice, and applied to slides that were denatured for 10 min in 70% FA, 2 × SSC, pH 7.0, at 72°C and dehydrated in a 70%, 90%, and 100% ethanol series. The slides were covered by a 24 × 50 mm^2 coverslip and sealed with rubber cement. Hybridization was performed at 37°C overnight in a moist chamber. After extensive washing of the slides in 50% FA, 2 × SSC, pH 7.0, at 42°C for 40 min, followed by 2 washes of 20 min each in PN buffer (0.1M sodium phosphate, pH 8.0, 0.1% NP40) at 37°C, the slides were incubated with 30 μl PNM buffer (PN buffer plus 5% non-fat dry milk and 0.02% sodium azide) for 10 min. Visualization of the bound biotin-labeled gamma satellite probe DNA was performed with yellow-green fluorescent avidin/FITC (5 μg/ml, Vector Laboratories, Burlingame, CA) in PNM buffer. The slides were incubated for 20 min at room temperature in a moist chamber and then washed twice in PN buffer at 37°C for 10 min each. The red fluorescent dye propidium iodide (2 μg/ml, PI, Sigma) and the blue fluorescent dye 4,6-diamidino-2-phenylindole (1 μg/ml, DAPI, Sigma), both in antifade solution [Johnson and de Nogueira Araujo, 1981], were used to counterstain the DNA. If necessary the hybridization signals were amplified by using biotinylated anti-avidin made in goat (Vector Laboratories) followed by another layer of avidin-FITC conjugate.

Scoring Procedure and Criteria

Microscopic analysis was performed on a Zeiss Axiophot microscope (Zeiss, Oberkochen, FRG) with fluorescence equipment using fluorescence filter sets 09 and 01 for the observation of PI/FITC- or DAPI-fluorescence, respectively.

Since the cytoplasmic membrane of the cells was not visible during microscopic fluorescence analysis, CB cells with well-preserved cytoplasm and a rounded shape were preselected prior to hybridization with a phase-contrast objective at 250× magnification. After relocation, cells were analyzed with a ×100/1.30 oil objective.

Cells were scored according to our previously published [Huber et al., 1989] and the following additional criteria: Both main nuclei and micronuclei had to be visible with PI and DAPI to exclude artefacts. The FITC signals had to be clearly visible and homogeneously dispersed within the main nuclei. Five-hundred CB cells per dose (for chemical and radiation exposure) were scored from at least two different cultures, except at the VBL 0.06 μM dose, in which, obviously due to a delay of nuclear division, only 154 scorable CB cells were available. In control slides 1,000 CB cells from five different cultures were analyzed. The MN analysis was performed by two scorers in uncoded slides. To avoid scorer bias, about 10% of the scored cells at each dose of VBL or radiation were relocated and analyzed by the second scorer. Since there were no substantial differences between the results the data were pooled.

RESULTS

Figure 1a,b shows that FITC centromere signals are clearly visible in main nuclei and in MN of CB cells. Figure 1d shows that in a colcemid-blocked metaphase spread the gamma satellite probe DNA hybridized to the centromeres of all chromosomes except the Y [Pietras et al., 1983]. Thus, presence of a hybridization signal in a MN is

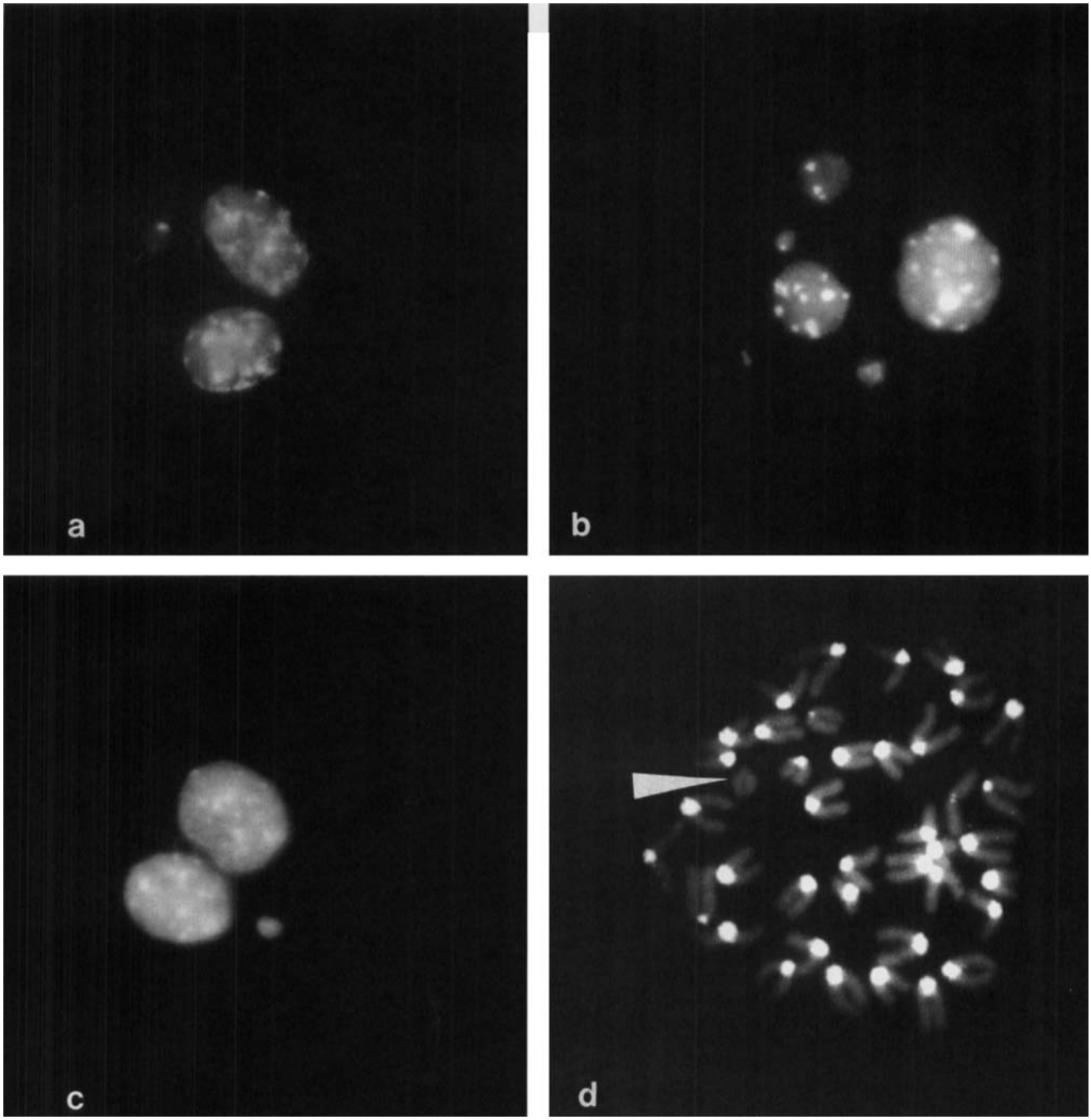

Fig. 1. Binucleate CB mouse liver cells after in situ hybridization with a biotinylated mouse gamma (major) satellite DNA probe. FITC centromeric DNA signals are visible in each of the main nuclei (a–c), in micronuclei **(a,b),** and on all metaphase chromosomes **(d)** except the Y chromosome (arrowhead). **c:** centromere-negative micronucleus.

a direct indication of the presence of a centromere (centromere-positive). Figure 1c shows a centromere-negative MN. When cells were exposed to gamma-rays or VBL, a dose-dependent increase in the number of micronucleated CB cells and MN per cell was observed for both agents (Table I, Figs. 2, 3). The total number of radiation-induced MN per cell could be fitted to a linear-quadratic model ($\chi^2 = 0.549$; df = 2; $p = 0.76$). CB cells containing more than one MN became apparent at higher doses. VBL-induced MN were centromere-positive in an order of

TABLE I. Frequency of Vinblastine- and Radiation-Induced Micronuclei in CB Mouse Liver Cells

Agent (dose)	No. of cells scored	Total No. of micronuclei	No. of CB cells with n micronuclei								Centromere-positive micronuclei (% of total No. of micronuclei)
			0	1	2	3	4	5	6	7	
Control											
0	1.007	35	973	33	1						29
Radiation (Gy)											
0.5	500	48	453	46	1						10
1.0	500	80	430	62	7	—	1				21
2.0	540	223	372	126	31	9	2				17
3.0	500	385	251	154	66	20	7	1	1		20
Vinblastine (μM)											
0.01	500	14	487	12	1						79
0.02	507	80	445	48	12	—	2				74
0.04	500	232	345	109	21	19	6				95
0.06	154	173	61	53	17	14	3	5	—	1	93

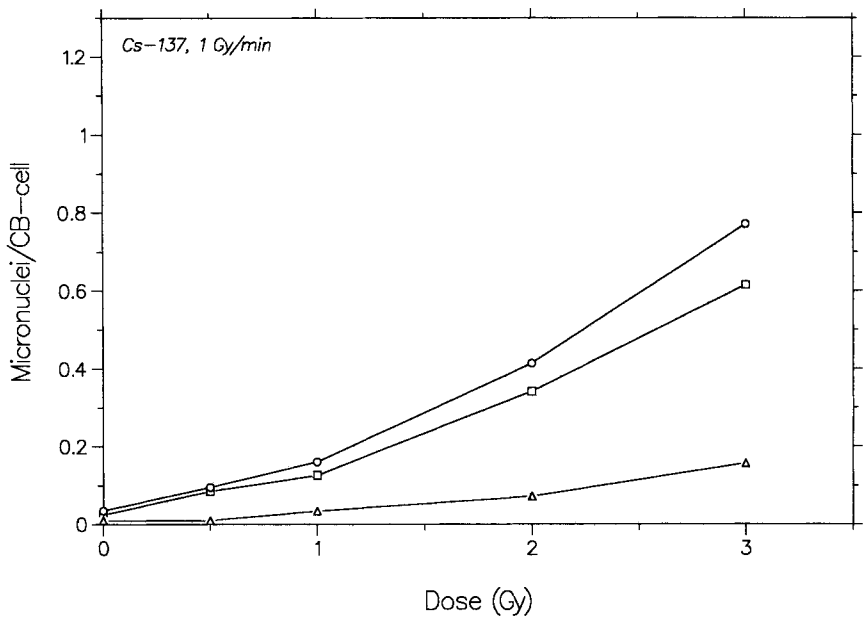

Fig. 2. Induction of micronuclei in mouse liver cells by ^{137}Cs gamma-rays. (○) total number of micronuclei per CB cell (Pearson correlation test: $p = 0.003$); (△) centromere-positive micronuclei ($p = 0.008$); (□) centromere-negative micronuclei ($p = 0.003$).

magnitude of 70–90%, but radiation-induced MN only in an order of magnitude of 10–20%. The corresponding dose-response curves are shown in Figures 2 and 3. In controls, 29% of MN were centromere-positive. Occasionally, in CB cells with multiple MN, centromere-positive and centromere-negative MN were observed (3–5 cells at 0.02–0.06 μM VBL, and 2, 9, and 32 cells at 1, 2, and 3 Gy gamma-rays, respectively).

DISCUSSION

The present findings of a dose-dependent induction of MN by the clastogen-ionizing radiation and by the aneuploidy-inducing agent VBL are consistent with previous results obtained in a CB micronucleus assay with human lymphocytes and Chinese hamster cells using an antikinetochore antibody for the identification of kinetochore-positive micronucleated cells [Eastmond and Tucker, 1989a,b]. Reported fractions of spontaneously occurring MN-containing kinetochores, determined by antikinetochore antibodies, were between 34 and 61% in human lymphocytes and fibroblasts [Eastmond and Tucker, 1989b; Fenech and Morley, 1989; Thomson and Perry, 1988; Cornforth and Goodwin, 1991] and between 48 and 79% in Chinese hamster cells [Eastmond and Tucker, 1989a]. This is slightly higher than our control value of 29% determined by ISH. Whether this reflects real differences in the capacity of either method to detect MN-containing centromeres

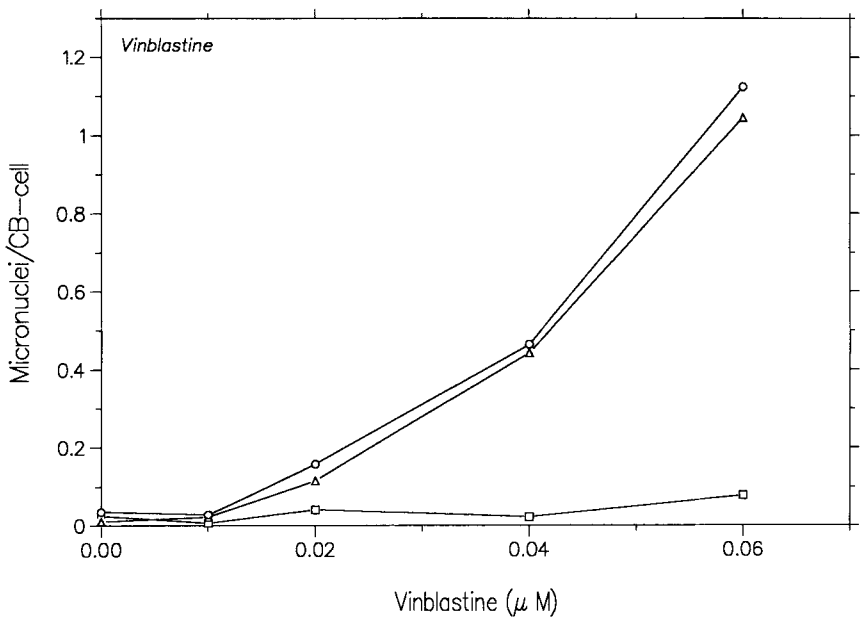

Fig. 3. Induction of micronuclei in mouse liver cells by vinblastine sulfate. (○) total number of micronuclei per CB cell (Pearson correlation test: $p = 0.012$); (△) centromere-positive micronuclei ($p = 0.010$); (□) centromere-negative micronuclei ($p = 0.150$).

cannot be decided since we have yet no data on the range of variation of our control level.

The proportion of radiation-induced kinetochore-positive MN is reported to be 3 to 14% in human lymphocytes and fibroblasts analyzed by CREST staining and was between 10 and 21% in the present study using ISH. These can be accepted to be in a similar range.

After VBL exposure of Chinese hamster cells Eastmond and Tucker [1989a] found 84 to 95% kinetochore-positive MN. This is in good agreement with the present findings of 74 to 95%. In our VBL experiment there was no evidence for an increase in the frequency of micronucleated CB cells after 0.01 μM exposure as compared to the control. However, the fraction of centromere-positive MN was considerably higher. This may be an indication for an initial spindle damage affecting single chromosomes which could be included in addition to acentric chromatin elements in the MN. Evidence for such a mechanism could come from the observation of average larger diameters of VBL-induced MN, although this has not been analyzed systematically.

Our results show that non-radioactive ISH using mouse major satellite DNA is a reliable alternative to the use of antikinetochore antibody for the discrimination between MN-containing centromeres, i.e., chromosomes, or acentric fragments. Despite the successful application of the CREST staining for classification of MN, several reasons, limiting its application for an unequivocal detection of centromeres, have been emphasized [Thomson and Perry, 1988; Becker et al., 1990]. It might be conceivable that the effect of specific kinetochore damage on CREST antibody binding which is mediated mainly by proteins is an important factor. This should not affect centromere detection by ISH since the gamma satellite DNA probe directly identifies centromeric DNA sequences. Mouse gamma or major satellite DNA is concentrated in the centromeric region of the mouse chromosomes and represents an estimated proportion of 5 to 10% of the murine genome [Weier et al., 1991]. Since only such clustered gamma satellite DNA repeats can be detected by ISH the method provides a specific visualization of centromeres. Due to their origin from different patients, no standardized CREST sera are available. Relevant gamma satellite DNA probes can, however, be generated repeatedly and in stable quality in the laboratory by polymerase chain reaction (PCR). CREST staining is less time-consuming than ISH, which is normally performed overnight. However, preliminary experiments have shown that adequate results can be obtained even after 5 hr of hybridization.

In conclusion, ISH with a biotinylated degenerate DNA probe of mouse gamma (major) satellite probe DNA can be reliably used in a CB MN assay to distinguish between an agent's clastogenic or aneuploidogenic capacity.

ACKNOWLEDGMENTS

The authors thank N. Gabellini for her skilful technical assistance.

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Accepted by—
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