A THREE WAY DIFFERENTIAL STAINING PROTOCOL FOR SCE ANALYSIS DURING THREE SUCCESSIVE CELL DIVISIONS IN MURINE BONE MARROW CELLS *IN VIVO*

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ABSTRACT

A method for the analysis of sister chromatid exchanges (SCEs) occurring in three subsequent cell divisions *in vivo*, was developed in murine bone marrow cells. The method was based on the three-way differential staining of sister chromatids. Mice were injected twice i.p. with BrdU adsorbed to activated charcoal; the first dose at 0 h (first cell cycle) was varied between 0.1 and 0.25 mg of BrdU/g of body weight and the second dose at 8 h (second and third cell cycles), was either 1.0, 1.33 or 1.5 mg of BrdU/g of body weight.

Animals were injected with colchicine (i.p.) two hours before sacrificing and chromosome prepations were obtained by the usual method. After sister chromatid differentiation by the method of fluorescence plus Giemsa the cells were scored for well and poor differential staining and in some cases for SCE frequency in three subsequent cell divisions.

The results indicate that a higher percentage of well-differentiated three way stained cells were obtained with the combination of BrdU doses of 0.2 and 1.5 mg/g of body weight. The very low basal frequency of SCE observed in the first cell cycle (0.31-0.46) probably increases the sensitivity to detected the mutagenic effect by SCE method. This method can be used for both the analysis of *in vivo* persistence of lesions involved in SCE induction as well as the detection of sinergism between the mutagen action and the BrdU incorporation to DNA.

RESUMEN

Se describe un método *in vivo* para el análisis de intercambio de cromátidas hermanas (ICH) durante tres ciclos de división sucesivos. El método está basado en la tinción diferencial de las cromátidas hermanas en tres tonos. Los ratones fueron inyectados dos veces intraperitonealmente con bromodesoxiuridina (BrdU) adsorbida en carbón activado; la primera dosis al tiempo cero (primer ciclo celular) se varió entre 0.1 y 0.25 mg de BrdU por gramo de peso y una segunda dosis a las 8 horas (segundo y tercer ciclos celulares) con 1.0, 1.33 ó 1.5 mg de BrdU por gramo de peso corporal.

Los animales fueron inyectados con colchicina (i.p.) dos horas antes de su sacrificio a las 35 h. Las laminillas obtenidas por la técnica usual fueron teñidas siguiendo el método de fluorescencia más Giemsa. Se cuantificaron las células con buena o pobre diferenciación y en algunos casos la frecuencia de ICH, en tres divisiones subsecuentes.

Los resultados indican que el mayor porcentaje de células con sus cromátidas hermanas teñidas en tres tonos se obtuvo con una combinación de dosis de 0.2 y 1.5 mg de BrdU/g de peso para la primera y segunda dosis, respectivamente. La frecuencia de ICHs tan baja

que se observó en el primer ciclo de división (0.31-0.46) probablemente aumente la sensibilidad para detectar el efecto mutagénico mediante el análisis de ICH. Este método puede ser usado para determinar *in vivo* la persistencia de lesiones involucradas en la producción de ICH y el efecto sinérgico entre la acción de los mutágenos y la BrdU incorporada al ADN.

INTRODUCTION

The induction of SCEs is considered a good index of the genetic damage caused by the exposition to mutagens (Latt *et al.* 1981). The phenomenon of sister chromatid exchange (SCE), originally demonstrated by autoradiography (Taylor 1958), has been amply studied since the development of techniques for the differential staining of sister chromatids utilizing bromodeoxyuridine (BrdU) (Latt 1974, Perry and Wolff 1974).

The protocol commonly used for the differential staining of sister chromatids involves the addition of BrdU during at least the first of two successive cell divisions. The SCE is analyzed in the metaphases of the second cell division, assuming that the frequency of the SCEs obtained is the sum of that occurred during the first and second divisions.

Through use of the technique of three-way differential staining of sister chromatids (TWD), it is possible to determine the frequency of SCE occurring in each of the three successive cell cycles. Two protocols for TWD *in vitro* have been reported. In that of Miller (1976), BrdU in decreasing quantities is added during the first two cell cycles and the cells are analyzed in the third division; whereas, in the method of Schvartzman and Goyanes (1980), BrdU is added in low concentrations during the first cell cycle and in higher concentration during the last two divisions. The later procedure has been used to analyze the persistence of lesions which induce SCE (Schvartzman *et al.* 1984), to evaluate the effect of BrdU incorporation to DNA on SCE induction and to estimate the effect of the BrdU incorporation to DNA on SCE production by mutangens (Morales-Ramírez *et al.* 1987). A modification of this *in vivo* procedure was used to stablish the persistence of mutagen-induced lesions involved in SCE production (Morales-Ramírez *et al.* 1988).

Herein, we report the adaptation of TWD technique (Schvartzman and Goyanes 1980) to murine bone marrow cells *in vivo* using BrdU adsorbed by activated charcoal.

MATERIALS AND METHODS

Animals. Two to three months old male Balb/C mice weighing between 30 to 35 g, were used. The animals were maintained in a temperature controled room and fed *ad libitum* with Laboratory Purina Chow.

BrdU-activated charcoal. BrdU was adsorbed by activated charcoal by mixing a BrdU solution (20 mg/ml) with activated charcoal (200 mesh) for 5 min, similar to the method previously described (Morales-Ramírez *et al.* 1984a).

For three-way differential staining of sister chromatids (TWD), mice were injected twice (i.p.), with combinations of BrdU doses; the first dose, at 0 h, was either 0.1, 0.16, 0.2 or 0.25 mg of BrdU per gram of body weight, the second dose, at 8 h, was 1.0, 1.33 or 1.5. The animals were injected with colchicine 15 μg per g of body weight (i.p.)

two hours before sacrifice at 35 h. The time of sacrifice was chosen to obtain a higher percentage of cells in the third division, assuming an average generation time of 12.4 h (Morales-Ramírez *et al.* 1984b).

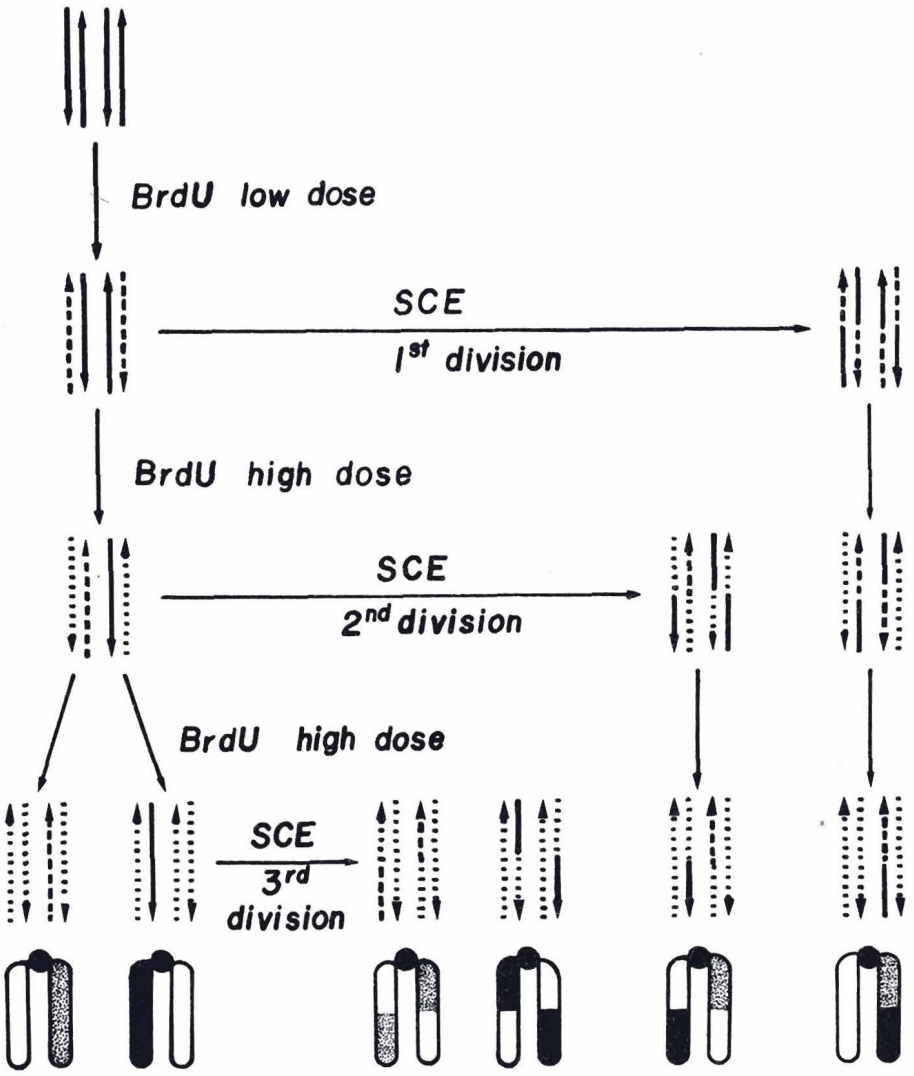

Fig. 1. Protocol for three-way differential staining of sister chromatids proposed by Schvartzman and Goyanes (1980). DNA strands with high (dotted lines), low (dashed lines), or no substitution (solid lines) with BrdU are represented, as well as the sister chromatid differential staining obtained when SCEs occured in the first, second and third cell cycle

Staining and SCE analysis. The bone marrow metaphases were obtained as was reported above (Morales-Ramírez *et al.* 1984a). The sister chromatids were differentially stained by using a modification (Goto *et al.* 1975) of fluorescence plus Giemsa technique (Perry and Wolff 1974). The SCE was analyzed in 30 cells per mouse and the exchanges that had occurred in the first, second and third cell division were determined using the protocol described by Schvartzman and Goyanes (1980) (Fig. 1).

The quality of three-way differential staining was determined from the analysis of two hundred metaphases per mouse, in at least four animals, applying the following criteria: poorly differentiated (PD) metaphases in which the sister chromatids were differentially stained but not sufficiently contrasted to permit the SCE analysis, and well differentiated (WD) metaphases in which the contrast between sister chromatid allowed SCE analysis.

RESULTS AND DISCUSSION

To determine the ratio between the first (first division) and second BrdU dose (second and third divisions) which permits the most efficient three-way differential staining of the chromatids, different combinations of doses were tested. The high doses used here were at least the minimun (1 mg per g weight) required for two-way differential staining *in vivo* in bone marrow cells when using BrdU-activated charcoal method (Morales-Ramírez *et al.* 1984a). These doses were used to obtain a better contrast between the unstained and the darkly stained chromatids, thereby increasing the range to better distinguish the intermediate tone.

The metaphase figures of third division were classified as well differentiated (WD) or poorly differentiated (PD) sister chromatids. In the latter category were included metaphases in which the three tones were not sufficiently contrasted to allow SCE analysis. In the experiments, approximately 20 to 50% of the cells in third division were found to have mitotic figures with only two tones. This was probably due to: the particular generation time of these cells, the duration of BrdU disponibility, the efficiency of BrdU incorporation or a combination of these possibilities. Only the cells with well or poorly differentially stained chromatids were considered for the estimate of the efficiency of differential staining.

The relation of dose coefficient (second/first) to the quality index (WD/PD) was not proportional (Fig. 2). The maximum quality was obtained at a dose coefficient of aproximately 7.5, and the optimal doses were 0.2 mg BrdU per gram weight for the first and 1.5 mg per gram weight for the second.

The frequencies of SCE in the first, second and third cell cycles for the different combinations of doses are shown in Table I. The basal frequency of SCE was very low in the first cycle, which possibly will permit a notable increase in the sensitivity for detecting the mutagenic effect of genotoxic agents. A direct relation exist between the doses of BrdU administered in the first and second cycles and their respective SCE frequencies, this observation suggest that most if not all of the SCE were induced by BrdU as was previously reported (Morales-Ramírez *et al.* 1987).

It was found that by using this in *in vivo* procedure, a high percentage of cells having their chromatids clearly differentiated in three tones can be obtained (Fig. 3). The suggested protocol consists of a first dose of 0.2 mg BrdU (previously adsorbed by activated charcoal) per gram of body weight at zero time, a second dose of 1.5 mg BrdU

per gram of body weight eight hours later, and an injection of colchicine (15 μg per gram weight) at 33 h, and the sacrifice of the animals two hours later.

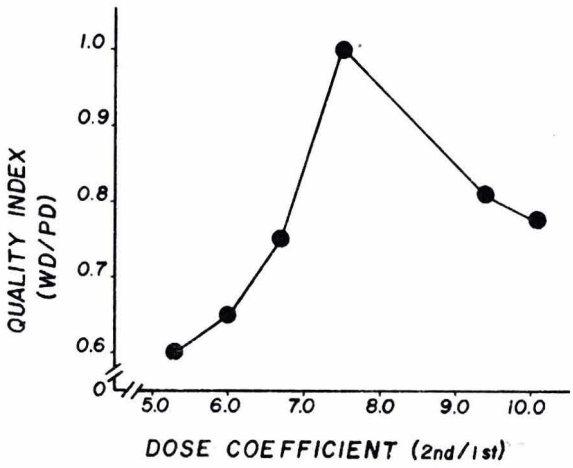

Fig. 2. Graph of the three-way differentiation quality index (well differentiated/poorly differentiated)versus the dose coefficient (2^{nd} dose/1^{st} dose). Each point represents the average of values from four to six animals

TABLE I. EFFECT OF BrdU DOSE ON THE FREQUENCY OF SCE IN THE FIRST, SECOND, AND THIRD CELL DIVISIONS

	BrdU Dose mg/g weight		SCE/cell/mouse (X±SD) Cell Division		
Number of mice*	1^{st}dose	2^{nd}dose	1^{st}	2^{nd}	3^{rd}
4	0.20	1.5	0.31±0.20	2.3±1.00	3.1±1.30
16	0.25	1.5	0.40±0.15	2.3±0.78	3.0±0.66
8	0.25	1.0	0.46±0.12	1.7±0.30	2.6±0.60

*Thirty cells were scored per animal

This *in vivo* procedure may be used to stablish the persistence of DNA lesions eliciting SCE (Morales-Ramírez *et al.* 1988), and to determine the effect that BrdU incorporation to DNA has on the sensitivity to SCE induction by mutagens (Morales-Ramírez in preparation).

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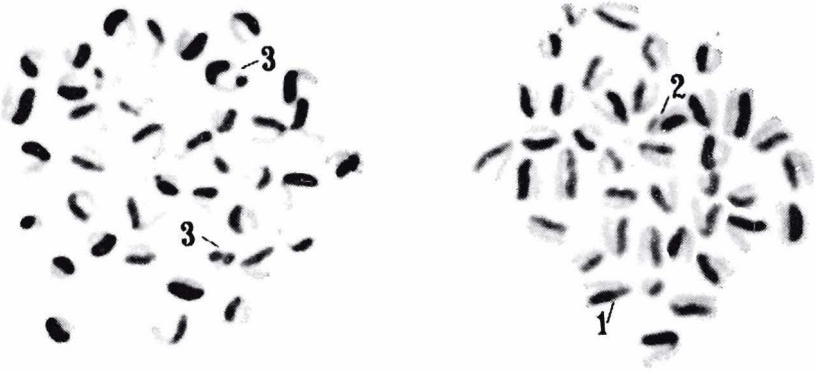

Fig. 3. Mouse bone marrow metaphases showing three-way differentially stained chromatids. SCE occurring in successive cell divisions are indicated (see Fig. 1)

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