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Induction of micronuclei and sister chromatid exchange in mouse splenocytes after exposure to the butadiene metabolite 3,4-epoxy-1-butene

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3,4-Epoxy-1-butene (EB) is one of the main metabolites of 1,3 butadiene, a widely used industrial chemical. The mutagenic potential of 1,3 butadiene and its metabolites have been studied in different test systems. In this work the genotoxic effects of EB were studied by estimating micronuclei (MN) and sister chromatid exchange (SCE) frequencies in stimulated mouse splenocytes. Mice were treated *in vivo* with various doses of EB (24.4, 48.8 and 73.2 mg/kg). The antikinetochore antibody technique (CREST) was also applied to MN in cytokinesis blocked cells to investigate any possible aneugenic effect. Both MN and SCE frequencies increased after EB treatment. The induced MN resulted mainly from acentric fragments but a weak aneugenic effect was found as well. Cytotoxic effects of EB were observed at the highest dose. The above results, in combination with others on the effect of 1,3 butadiene and its metabolites in somatic and germ cells of mouse and rat as well as in somatic human cells, form a part of the information needed for application of the parallelogram approach and extrapolation to human risk.

Introduction

During the last decade great effort has been expended in order to evaluate the mutagenic and carcinogenic potential of 1,3 butadiene (BD) and its metabolites. BD is one of the world's major industrial gaseous chemicals, colorless and flammable, that is used in the production of polymers and synthetic rubber. It is also detectable in urban air, car exhausts and cigarette smoke (IARC, 1992). As an environmental contaminant it has been extensively studied. Occupational exposure of workers to high air concentrations of BD has been shown to be associated with the development of tumors of the hematopoietic system (Landrigan, 1993). Although a small number of studies have been published referring to the effect of BD in humans occupationally exposed, it seems that there is no correlation between exposure and increase in various cytogenetic parameters, such as chromosomal aberrations (CA), sister chromatid exchange (SCE) and micronuclei (MN). However, in a recent study of BD genotoxicity in workers from a plant in Prague, analysis of chromosomal aberrations in lymphocytes indicated that the percentage of aberrant cells was significantly enhanced in exposed subjects (Tates *et al.*, 1996).

In contrast, *in vivo* studies in somatic cells have indicated that BD is clastogenic, inducing increased frequencies of SCE, CA and MN in mice (Cunningham *et al.*, 1986; Autio *et al.*, 1994) but not in rats (Autio *et al.*, 1994). BD is therefore characterized as a species-specific mutagen. Results from *in vivo* mutagenicity studies in somatic cells demonstrate that BD is a somatic mutagen (Adler *et al.*, 1995). BD may also be considered as a germ cell mutagen, since it has been shown to induce heritable translocations (Adler *et al.*, 1995) and dominant lethal effects (Adler and Anderson, 1994) as well as increased MN frequencies in germ cells of mice (Xiao and Tates, 1995). 3,4-Epoxy-1-butene (EB) and 1,2,3,4-diepoxybutane (DEB) are the two major metabolites of 1,3 butadiene. Different results have been obtained in experiments on mice and rats in relation to these metabolites. Although it has been demonstrated that BD is metabolized in mouse and rat by the same pathway (Thorton-Manning *et al.*, 1995), different concentrations of these metabolites may be found in mouse and rat tissues (Bond *et al.*, 1995), EB being detected in greater quantities in mouse than in rat. Mice metabolize BD to EB faster than rats but have a limited capacity for detoxification and accumulation of the reactive epoxide intermediate, thus explaining the greater susceptibility of mice to BD (Bolt, 1993).

EB has been reported to be mutagenic in bacteria (Gervasi *et al.*, 1985), to induce SCEs in mammalian cells (Sasiadek *et al.*, 1991a,b) and also to increase MN frequencies in somatic and germ cells in mice and rats (Xiao and Tates, 1995; Russo *et al.*, 1997). It has also been reported to be mutagenic at the *tk* and *hprt* loci of cultured human lymphoblasts and at the *hprt* locus of mouse splenic T cells (Cochrane and Skopek, 1994a,b).

The cytokinesis block MN assay applied to mouse splenocytes has been shown to be a useful tool in estimation of chromosome damage, comparable with the human lymphocyte MN assay (Fenech *et al.*, 1991; Ren *et al.*, 1991; Benning *et al.*, 1994; Renzi *et al.*, 1996). MN induced by different compounds can be discriminated with respect to their content (acentric chromosome fragments or a whole chromosome) by applying anti-kinetochore antibodies in combination with immunofluorescence techniques (Degrassi and Tanzarella, 1988; Eastmond and Tucker, 1989; Miller and Adler, 1990; Norppa *et al.*, 1993; Renzi *et al.*, 1996). The SCE assay has been considered a good indicator of the mutagenic potential of several chemical agents and has also been applied in murine splenocytes (Krishna *et al.*, 1988; Darroudi *et al.*, 1992; Kligerman *et al.*, 1993).

This study forms part of a Joint European Union Project (EV5V-CT94-0543) for the evaluation of the genotoxicity of BD and its metabolites in somatic and germ cells of different species, thus providing the necessary information for application of the parallelogram approach (Sobels, 1982; Anderson *et al.*, 1994; Pacchierotti *et al.*, 1997). The concept of the parallelogram approach is that an estimate of the genetic damage in human germ cells can be obtained by measuring a common end point in human and mouse somatic cells and in germ cells of mice, the desired target tissue, which is inaccessible in humans (Waters

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and Nolan, 1995). The aim of this study was to further investigate the ability of EB to induce MN and SCE in mouse somatic cells, namely splenocytes, and also to explore the possibility of an aneugenic effect, since recently it was reported that BD and its metabolites EB and DEB are weak aneugens in mice (Xiao *et al.*, 1996).

Materials and methods

Animals and chemicals

BALB/c mice were purchased from Charles River (Calco, Lecco, Italy) and during the treatment they were 8–12 weeks old. Mice were housed for at least 1 week under the new conditions, i.e. 21–23°C, 40–50% humidity, 12 h dark/light cycle, before treatment. EB (CAS no. 930-22-3; Sigma-Aldrich) was freshly diluted in Hank's balanced salt solution (HBSS) and injected i.p. immediately after dilution in a volume corresponding to 0.01 ml/g body wt. Control mice were injected with HBSS. Three different doses were studied, 24.4, 48.8 and 73.2 mg/kg. Since this work is part of a joint EU project and the same mice were used for the study of EB effects on germ cells and peripheral reticulocytes (Russo *et al.*, 1997), the doses and the time of splenocyte isolation were selected in accordance with the general schedule. G.S. and C.A. (Patras) visited Padova to set up splenocyte cultures and prepare the apporpriate slides, while SCE, MN scoring and CREST analysis were done in Patras.

Splenocyte isolation

Animals were killed 2 and 16 days after treatment by cervical dislocation. Spleens were dissected from each animal and splenocytes were collected by forcing the spleen through a sterilized nylon filter and washing the filter to collect the cells in phosphate-buffered saline (PBS). The cell suspension was then gently layered on the same volume of Histopaque 1077 (Sigma) and centrifuged at 800 *g* for 25 min. The interface was carefully collected, resuspended in PBS and centrifuged at 200 *g* for 15 min. The cell pellet was once more resuspended in PBS and centrifuged at 200 *g* for 10 min. Finally, the cell pellet was resuspended in 1 ml PBS. The cell concentration was estimated using a hemocytometer.

Cultures

Splenocyte cultures were initiated at a concentration of 1×10^6 cells/ml in complete medium. Growth medium consisted of RPMI 1640 (Seromed, Berlin, Germany) supplemented with 15% fetal calf serum (Biospa, Wedel, Germany), 2 mM L-glutamine and antibiotics. Concanavalin A (Sigma) at a final concentration of 5 mg/ml was used as mitogen. Cultures were allowed to grow at 37°C in a 5% CO_2 atmosphere with 95% humidity.

Micronucleus analysis

For the MN study 3 μg/ml cytochalasin B (Sigma) was added 18 h before cell harvesting. Cells were harvested 44 h after culture initiation. Cells were collected by centrifugation at 200 *g* for 10 min. The supernatant was removed and the cell pellet resuspended in fresh medium. Slides were prepared in a Cytocentrifuge (Shandon, UK) at 400 r.p.m. for 5 min, air dried and fixed in absolute ethanol at –20°C. Slides can be stored in ethanol at –20°C for up to 2 months without any detectable loss of antigenicity of the kinetochores.

Microscope slides with splenocytes from the cytospin were rehydrated in PBST 0.1 (0.1% Tween 20 in PBS) for 10 min and then incubated with CREST serum (Davis, CA) diluted 1:1 in PBST 0.2 for at least 1 h at 37°C in a moist chamber. After the end of incubation the unbound antibodies were removed by rinsing the slides twice in PBST 0.1 for 10 min each. FITC-conjugated anti-human polyvalent immunoglobulins (1:100 in PBST 0.1; Sigma, Immunochemicals) were laid onto the slide, allowed to link with CREST antibodies and incubated under the same conditions as above. The slides were then rinsed with PBS several times for at least 5 min each and counterstained with ethidium bromide in Vectashield mounting medium (Vector Laboratories) at a final concentration of 2 μg/ml. Slides were kept in the dark at 4°C and analyzed in a Zeiss Axioskop fluorescence microscope equipped with bandpass filters of 546 and 490 nm, for green and blue light respectively. For determination of MN frequencies at least 1000 binucleated cells were analyzed for each experimental point and for each mouse. For identification of MN standard criteria were followed. At least 50 MN were analyzed for the presence of a kinetochore for each experimental point. To identify any MN as containing a CREST-positive signal this should be of the same intensity as those of the main nuclei.

SCE analysis

For analysis of SCE 5-bromo-2-deoxyuridine (Sigma) was added to the culture medium at a final concentration of 5 μM when the cultures were set up. Three hours before cell harvesting Demecolcine (Sigma), at a final concentration of 0.3 μg/ml, was added to the culture to arrest metaphase. Cells were harvested 48 h after culture initiation. Cells were collected by centrifugation at 200 *g* for 10 min, the supernatant discarded and the cell pellet gently resuspended in an equal volume of fresh 0.075 M KCl hypotonic solution, prewarmed to 37°C, and incubated at 37°C for 25 min. At the end of incubation the cells were centrifuged at 200 *g* for 10 min, the supernatant removed, the cells resuspended in an equal volume of fresh fixative and stored at 4°C for 10 min. Two more fixations followed and the concentrated cell suspension was dropped onto wet slides and then left to air dry. After 2 days the slides were processed by a modification of the fluorescence plus Giemsa technique to obtain harlequin stained chromosomes. They were incubated for 15 min in a solution of 0.5 μg/ml Hoechst 33258. After that they were washed and then placed under a UV illuminator in SSC for at least 1 h. At the end of that time slides were washed and stained for 10 min in 3% Giemsa solution in Sorensen's buffer, pH 6.8. One hundred second metaphases were analyzed for estimation of SCE frequencies at each experimental point wherever possible. The replication index was also calculated to determine any cell cycle delay. It was calculated according to the formula $RI = (M1 + 2M2 + 3M3)/n$, where M1, M2 and M3 correspond to those metaphases in the first, second and third or more divisions and n corresponds to the total number of metaphases scored (Lamberti *et al.*, 1983).

Statistical analysis

Statistical analysis of MN data was made by the G-test for independence on 2×2 tables (Sokal and Rolf, 1981). This test is based on the general assumption of the χ^2 analysis, but offers theoretical and computational advantages. The SCE were statistically analyzed by Student's *t*-test.

Results

The results of our study are presented in Table I. An increase in MN frequency is observed only at the dose of 48.8 mg/kg. As can be seen in Table I, at the dose of 48.8 mg/kg a statistically significant increase in MN frequency is apparent at both experimental times, 2 and 16 days after treatment, i.e. 15.74 and 13.56‰ respectively. As can be seen in Table I, the percentage of binucleated cells (BN) is reduced at the higher tested dose. The variability observed in BN proportion at the same experimental point could be attributed to culture-to-culture variations in the percentage of cells responding to the mitogen. Figure 1 shows the MN and micronucleated BN frequency.

CREST analysis has shown that most MN induced at the dose of 48.8 mg/kg were mainly composed of acentric chromosome fragments, but it seems that EB can also express a weak aneugenic action. As can be seen in Table I, the percentage of K^+MN, i.e. MN containing kinetochores, is lower or at least at the same level, 40.98 and 38.38% compared with 45.35 and 38.36% for controls, at 2 and 16 days after treatment respectively, indicating clastogenic activity. However, the K^+MN frequencies, 6.45 and 5.20, are higher and significantly different in relation to control frequencies, 3.88 and 3.07, at both time intervals, indicating a weak aneugenic effect. The same is also true for the K^-MN frequencies, 9.29 and 8.34, for the two experimental times, confirming the clastogenic activity of EB shown by the K^+MN percentage. The above results are depicted in Figure 2.

Table II presents the effect of EB on SCE formation. At the 2 day time interval a significant increase in SCE frequency is observed. For the two higher doses, 48.8 and 73.2 mg/kg, the observed mean SCE values are 10.90 ± 0.43 ($P < 0.05$) and 13.8 ± 0.51 ($P < 0.05$) respectively, which are statistically significantly different from controls. At the same experimental time interval the RI observed at the dose of 73.2 mg/kg (1.57) indicates a strong cell cycle delay. At the second time interval, 16 days after treatment, a significant increase in SCE frequency was found for the two higher doses, 8.13 ± 0.32 ($P < 0.05$) and 7.47 ± 0.28 ($P < 0.05$), although it was less pronounced with respect to the increase observed at the 2 day time interval,

Table I. MN frequencies estimated per 1000 BN and kinetochore-positive (K^+MN) and kinetochore-negative (K^-MN) MN as evaluated by CREST analysis in mouse splenocytes cultured 2 and 16 days after *in vivo* treatment with different doses of EB

Dose (mg/kg)	Number of mice	Time (days)	BN scored	MN	MN (‰)	K^+MN	% K^+MN	K^+MN (‰)	K^-MN	K^-MN (‰)	BN (%)
0	3	2	10 051	86	8.56	39	45.35	3.88	47	4.68	19.70–37.75
0	3	16	9125	73	8.00	28	38.36	3.07	45	4.93	35.20–38.40
24.4	5	2	9213	91	9.88	25	27.47	2.71	66	7.16	14.23–29.93
24.4	3	16	6105	65	10.65	28	43.08	4.59	37	6.06	35 45–46.10
48.8	5	2	7749	122	15.74[a]	50	40.98	6.45[b]	72	9.29[a]	18.05–42.27
48.8	5	16	7314	99	13.56[c]	38	38.38	5.20[b]	61	8.34[c]	28.15–35.40
73.2	5	2	5886	56	9.51	25	44.64	4.25	31	5.27	4.92–20.04
73.2	4	16	8708	74	8.50	31	41.89	3.56	43	4.94	8.78–31.28

[a]$P < 0.0001$; [b]$P < 0.05$, [c]$P < 0.001$.
BN, binucleated cells; MN, micronuclei; K^+MN, kinetochore-positive micronuclei; K^-MN, kinctochore-negative micronuclei.

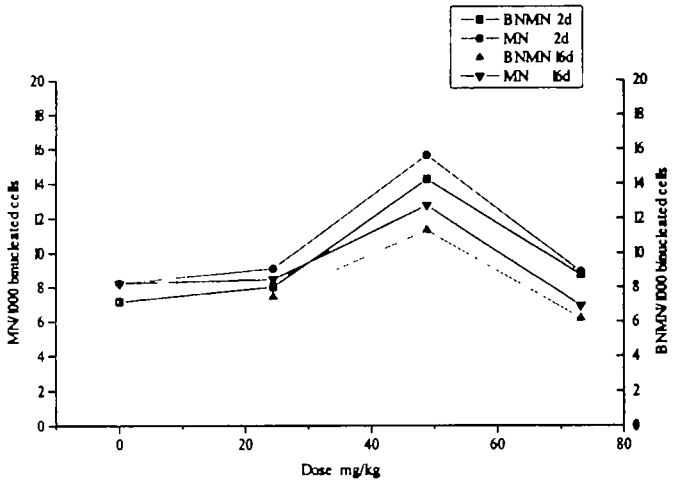

Fig. 1. Total data on frequencies, per thousand BN, of MN and micronucleated binucleates (BNMN) induced in mouse splenocytes cultured 2 (2d) and 16 (16d) days post-treatment with different doses of EB.

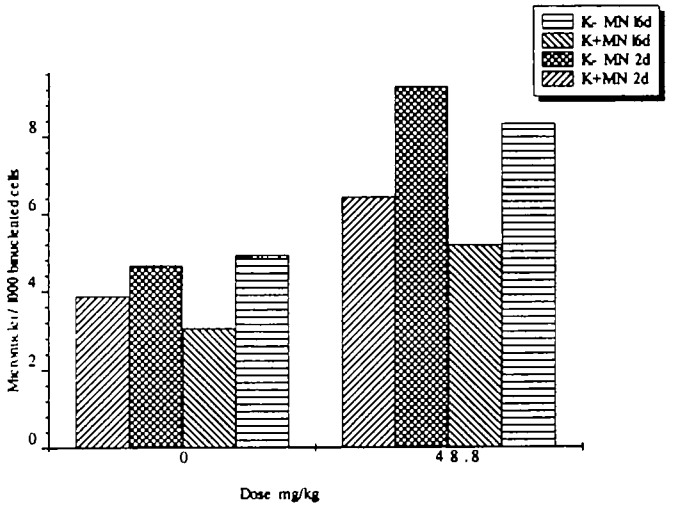

Fig. 2. Summarized results evaluated by CREST analysis in mouse splenocytes after *in vivo* treatment with 48.8 mg/kg EB. Kinetochore-positive (K^+MN) and kinetochore-negative (K^-MN) frequencies were estimated per thousand BN at 2 (2d) and 16 (16d) days post-treatment.

as is shown in Figure 3. No cell cycle delay was observed at that time interval. The change in SCE distribution with time post-treatment is depicted in Figure 4. A reduction in SCE response is seen with time.

Discussion

In our study an effort has been made to determine the genotoxic effects of EB, a major metabolite of BD in mouse splenocytes treated *in vivo*, by estimating MN and SCE frequencies. Our results show that EB is able to induce MN in mouse splenocytes treated *in vivo*. Statistically significant MN as well as micronucleated BN frequencies are induced after treatment of mice with 48.8 mg/kg at both time intervals (Figure 1). In a previously published study (Xiao and Tates, 1995) EB was tested on mouse splenocytes at doses of 40 and 80 mg/kg and it was found that MN frequencies were significantly increased at 80 mg/kg compared with those from the dose of 40 mg/kg at 1 and 3 days after treatment. We also observed that MN are increased at about the same dose and that this increase is still observed when splenocytes are stimulated 16 days after *in vivo* treatment.

Using CREST antibodies we found that the induced MN are mainly composed of acentric chromosome fragments, since the proportion of K^+MN is below the control value at both times, thus indicating that EB acts as a clastogen in mouse splenocytes. However, possible aneugenic activity of EB could not be excluded and this is supported by the fact that when frequencies of MN are calculated separately for the two categories (K^+MN and K^-MN) on the number of cells scored and these frequencies are compared with the corresponding control values, at the dose of 48.8 mg/kg an increase in K^+MN is observed as well as an increase of K^-MN (Figure 2). These results are in agreement with those of Xiao *et al.* (1996), who found by FISH experiments with a centromeric probe that besides its pronounced clastogenic effect in splenocytes of mice, EB is also a weak aneugen.

EB appeared cytotoxic, as far as the BN are concerned, and this could explain the lack of MN increase at the highest tested dose. A reduction in RI was also observed at the same dose. A cytotoxic effect of EB was also found in peripheral blood reticulocytes, in a subgroup of the same treated mice used in this study (Russo *et al.*, 1997).

EB has recently been identified as a weak genotoxic agent in germ cells of mice and rats (Xiao and Tates, 1995; Russo *et al.*, 1997). In a different somatic cell population of a group of the same mice used in the present experiments, namely bone marrow cells, EB induced MN at the three doses tested, with the maximum effect found at the highest dose, where a 9-fold increase was observed in peripheral blood reticulocytes (Russo *et al.*, 1997). Again, the cytotoxicity of the compound can explain the different responses of the two compartments,

Table II. SCE frequencies in mouse splenocytes cultured 2 and 16 days after *in vivo* treatment with different doses of EB

Dose (mg/kg)	Number of mice	Time (days)	Number of cells analyzed	SCE/cell (mean ± SE)	Range	*t* value (compared with control)	*P* value	RI
0	2	2	100	7.22 ± 0.31	1–16			2.20
0	2	16	100	6.61 ± 0.23	2–13			2.10
24.4	2	2	100	7.80 ± 0.28	3–15	1.36	>0.05	2.15
24.4	2	16	100	7.20 ± 0.27	2–15	1.67	>0.05	2.01
48.8	2	2	100	10.90 ± 0.43	2–26	6.87	<0.05	1.90
48.8	2	16	100	8.13 ± 0.32	3–18	3.83	<0.05	2.05
73.2	2	2	68	13.80 ± 0.51	6–25	11.56	<0.05	1.57
73.2	2	16	100	7.47 ± 0.28	1–14	2.36	<0.05	2.15

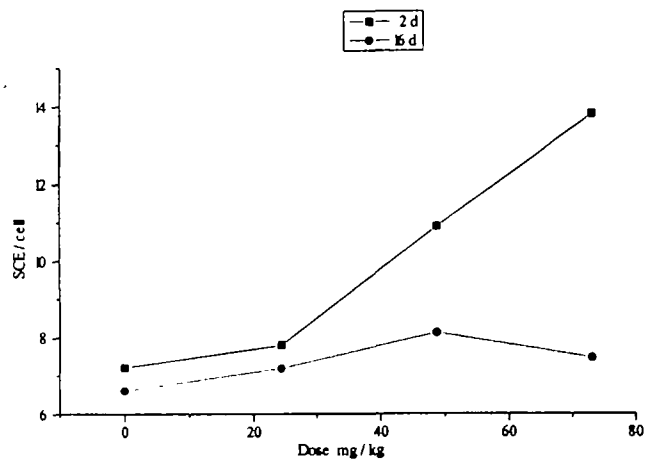

Fig. 3. SCE induced by different doses of EB in mouse splenocytes treated *in vivo* and cultured 2 (2d) and 16 (16d) days after treatment.

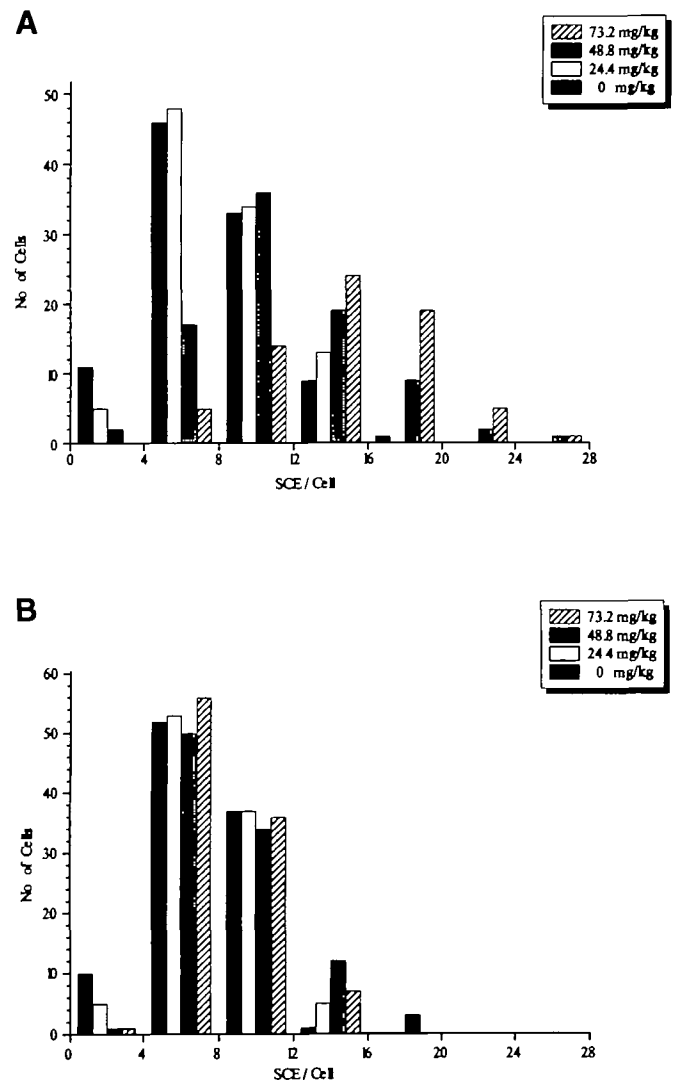

Fig. 4. SCE distribution in mouse splenocytes after *in vivo* treatment with different doses of EB cultured 2 (A) and 16 (B) days after treatment. The *x*-axis shows the classes of cells with the indicated number of SCE per cell (0–3, 4–7, 8–11, 12–15, 16–19, 20–23 and 24–27) and the *y*-axis shows the number of cells in a given class.

which are characterized by diverse replicative and differentiative activities. In addition, it should be noted that the MN assay on peripheral blood reticulocytes intrinsically has high sensitivity since internal negative controls are used, thus lowering the problems related to inter-individual variability. However, Sjöblom and Lähdetie (1996) found that EB studied *in vitro*, at concentrations ranging from 100 to 1000 μM, did not cause any increase in MN frequency in rat spermatids and at the higher concentration cytotoxic effects were determined, while from the same laboratory *in vivo* results on EB were similar to those of Xiao and Tates (1995). These contradictory results may be explained by the possible need for oxidation of EB to DEB in order to express its genotoxic action (Sjöblom and Lähdetie, 1996).

The study of the effect of EB on induction of SCE in mouse splenocytes showed that EB is also an inducer of SCE. Significantly higher SCE frequencies were determined only for the higher doses, 48.8 and 73.2 mg/kg, at 2 as well as 16 days after treatment (Figure 3). However, mean SCE values were lower at 16 than 2 days post-exposure and this was also observed for MN frequency. In addition, the SCE distribution with time post-treatment (Figure 4) showed a decrease in SCE response. At the 2 day time interval the majority of cells at the 48.8 and 73.2 mg/kg doses had 4–15 and 8–19 SCE respectively, while at 16 days post-treatment the majority of cells had 4–11 SCE at both doses. This decline in SCE demonstrates that dose and time after exposure are involved in determining lesion persistence. The same conclusions have been drawn by Sasiadek *et al.* (1991b), who reported that EB tested in CHO cells was highly effective in inducing SCE at concentrations of 0.1–1 μM in the presence and absence of S9 mix. This finding was also reported in human lymphocytes treated *in vitro* (Sasiadek *et al.*, 1991a). A cell cycle delay was also observed at 2 days, especially for the higher dose of 73.2 mg/kg, for which a lower percentage of binucleated cells was also observed.

Finally, the main conclusions of our study on the effect of EB in mouse splenocytes *in vivo* are: (i) the clastogenic

potential of EB has been demonstrared by enhanced frequency of MN in a resting cell population treated *in vivo* and EB also induced an increase in SCE frequency; (ii) the damage induced by EB is not persistent, since MN and SCE frequencies decrease at the second time interval; (iii) EB appeared to be a clastogenic agent with a weak aneugenic potential and also induced cytotoxicity.

The above results, in combination with others on the effect of BD and its metabolites in somatic and germ cells of mouse and rat as well as in somatic human cells, form a part of the information needed for application of the parallelogram approach and extrapolation to germ cell risk in humans.

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