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A Comparison of Baseline and Cyclophosphamide-Induced Sister Chromatid Exchanges in Bone Marrow and Spleen Cells of Mouse and Chinese Hamster

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Baseline sister chromatid exchange (SCE) frequencies were investigated in bone marrow and spleen cells of mice and Chinese hamsters. No significant difference in SCE frequency was noted for bone marrow in both species and for bone marrow and spleen in mice on per cell and per pg DNA basis. However, a significant difference was noted between species in spleen and between cell types in Chinese hamsters. Also, statistically significant differences were noted between species for both cell types when the same data were expressed on per chromosome basis. SCE levels in cultured bone marrow and spleen cells after intraperitoneal administration of the antineoplastic drug cyclophosphamide (10 and 20 mg/kg) differed significantly in mice and Chinese hamsters on per cell, per pg DNA content, and per chromosome basis. The spleen cells were much more sensitive to the effects of cyclophosphamide than bone marrow cells in both species. The replicative indices did not differ significantly between treated and control animals in either bone marrow or spleen cells of both species. Since SCE frequency is a sensitive measure of DNA damage, and bone marrow and lymphocytes are the most widely used cell types in human and animal in vivo assays, the methodologies and results reported here may be useful for comparative mammalian cytogenetic studies.

Key words: SCE, in vivo/in vitro primary cell culture, species sensitivity, replicative index, genotoxicity, cytogenetic assays

INTRODUCTION

In recent years, several short-term in vivo and in vitro tests have been developed for the identification of genotoxic agents [Hollstein et al, 1979]. Among these, sister

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chromatid exchange (SCE) has been demonstrated to be a sensitive measure of DNA damage [Perry and Evans, 1975; Latt et al, 1981; Carrano et al, 1978; Tice et al, 1984]. This assay has been adapted for both in vivo and in vitro studies in order to screen for genotoxic effects in specific target tissues and organs [Allen et al, 1977; Allen and Latt, 1976; Conner et al, 1979; Dearfield et al, 1984]. Several studies have demonstrated that SCE induction is correlated with gene mutation [Carrano et al, 1978; Krishna et al, 1984] and in vitro cell transformation [Popescu and DiPaolo, 1982] and can serve as a predictor of carcinogenic potential [Latt et al, 1981]. SCE analysis is also presently used to screen human populations for possible genotoxic effects resulting from occupational exposure [Archer et al, 1981].

Cyclophosphamide is an important antineoplastic and immunosuppressive agent. It is also a carcinogen in humans as well as in rodents [IARC Monographs, 1981]. In recent years, numerous cases have been reported regarding the occurrence of secondary neoplasias in cancer patients as well as cancers in noncancer patients treated with cyclophosphamide [Baltus et al, 1983; IARC Monographs, 1981]. Several other genotoxic activities have also been attributed to cyclophosphamide including mutagenicity [Ellenberger and Mohn, 1977], SCE inducibility [Dearfield et al, 1984; Takeshita and Conner, 1984], and clastogenicity [Schmid et al, 1971; IARC Monographs, 1981].

Numerous factors can influence the response of both laboratory animals and human subjects to exposed genotoxicants. Species, strain, and sex of the exposed individual are important and well recognized factors. Deposition patterns, distribution of absorbed materials, target organs for toxicity, and the threshold can alter the responses. Even though there have been several genotoxicity studies involving SCEs in different cell lines, tissues, strains, and species in vivo and/or in vitro, very few comparative studies among these have been made [Dearfield et al, 1984; Takeshita and Conner, 1984; Conner et al, 1978; 1979].

In this paper we report comparative results on baseline and cyclophosphamide-induced SCE frequencies in spleen and bone marrow cells of mice and Chinese hamsters. These results may help elucidate the differences among genotoxicity responses as measured by SCEs in spleen and bone marrow cells following in vivo exposure of mice and Chinese hamsters to cyclophosphamide. This study comprises validation of recently developed rodent cell culture systems for genotoxicity studies, which simulate human cytogenetic monitoring techniques and involve similar cell populations, ie, bone marrow and lymphocytes.

MATERIALS AND METHODS

Animals

Male, CD_1 mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). The male Chinese hamsters were from Chickline Industries (Vineland, NJ). All animals were 2–3 mo old and weighed 25–30 g. Mice and Chinese hamsters were housed separately in cages containing hardwood chip bedding and excelsior nesting material. They were acclimatized to our laboratory for 7–10 days after arrival. Water and Purina laboratory rodent chow were provided ad libitum throughout the period of animal holding and experimentation.

Drug Treatment

Cyclophosphamide (Sigma Chemical Co., St. Louis, MO) was dissolved in phosphate buffered saline (PBS) and immediately injected intraperitoneally in a

volume equivalent to 10 ml/kg body weight (10 and 20 mg cyclophosphamide/kg body weight). These concentrations were chosen for comparison because they caused a significant increase of SCEs in both mice and Chinese hamsters [Neal and Probst, 1983; Krishna et al, 1985]. Control animals were injected with an equivalent volume of PBS. Animals were killed 6 hr after injection by cervical dislocation.

Mouse and Chinese Hamster Spleen Cell Culture

Spleens from both species were obtained aseptically by opening the abdominal cavity, then transferred into centrifuge tubes, each containing 2 ml RPMI 1640 with L-glutamine and Hepes buffered medium (GIBCO, Grand Island, NY) supplemented with 20% heat-inactivated fetal bovine serum (FBS, GIBCO), 2 mM additional L-glutamine (GIBCO), and 1% penicillin-streptomycin (GIBCO). The spleen was smashed with the aid of a sterile spatula. The debris was removed, and cells were washed with PBS containing 2% heat-inactivated FBS three times by centrifugation at 285g for 6 min. Approximately 1.5×10^6 cells were cultured in 5 ml medium consisting of 3.70 ml RPMI 1640 with L-glutamine and Hepes buffer, 1.0 ml heat-inactivated FBS, 0.05 ml penicillin-streptomycin, 0.05 ml of 200 mM L-glutamine solution, 1×10^{-5} M 2-mercaptoethanol (Sigma), 20 μM BrdU (Sigma), and 0.2 ml of lipopolysaccharide (*Escherichia coli* serotype 0111:B4; Sigma, stock of 600 μg/ml in PBS). The cell suspension with the complete medium was dispensed into 25-cm^2 Falcon tissue culture flasks, covered with aluminum foil, and then incubated at 37°C with 98% relative humidity and 5% CO_2. After 40 hr incubation, colchicine (33 μM final concentration, GIBCO) was added, and cells were harvested 3 hr after colchicine treatment.

Bone Marrow Removal and Culture

The bone marrow culture procedure was described in a previous study [Krishna et al, 1985]. In brief, femora and tibia were removed from the same mice and Chinese hamsters used for spleen isolation. These bones were freed of adherent muscle and cleaned with 70% ethyl alcohol. The tips of the bones were then removed with scissors. The marrow was flushed out with Ham's F-12 medium (Flow Laboratories, McLean, VA) into a 15 ml centrifuge tube. Following removal of debris, the tubes were centrifuged at 285g for 6 min. The supernatant was removed, and the pellet was resuspended with the remaining solution. The mouse bone marrow cell suspension (approximately 1.5×10^6 cells) was incubated in 25-cm^2 Falcon flasks with 5 ml of the following complete medium: 3.45 ml Ham's F-12, 1 ml FBS (20%), 0.05 ml penicillin-streptomycin (1%), 0.5 ml pregnant mouse uterus extract (10%), and 20 μM BrdU. The Chinese hamster bone marrow cells were incubated in the same medium but without pregnant mouse uterus extract. Cultures were then covered with aluminum foil and incubated at 37°C and 98% relative humidity. After 30 hr incubation, colchicine was added, and cells were harvested 3 hr later.

Cell Harvest, Slide Preparation, and Staining

Contents of the flask were decanted into 15-ml Falcon centrifuge tubes. The flasks were rinsed with 3 ml Hank's balanced salt solution, which was then transferred to the centrifuge tubes. The tubes were centrifuged at 285g for 6 min. The supernatant was aspirated, and the cell pellet was resuspended in 5 ml hypotonic solution (0.075 M KCl at 37°C) for 20 min and recentrifuged. The cells were fixed twice, each time

with 5 ml freshly prepared 3:1, methanol:acetic acid. Finally, the cell suspension was diluted with a few drops of fixative and dropped on precleaned chilled wet slides, which were then air-dried for 24 hr.

Staining for SCE analysis was performed according to a modified technique of Perry and Wolff [1974] and Goto et al [1978]. Slides were stained for 15 min with Hoechst 33258 (5 μg/ml) and exposed to "black" light at 55–60°C for 15 min at a distance of 1 cm while immersed in Sorenson's buffer (phosphate buffer, pH 6.8). The slides were then rinsed with distilled water and stained with 5% Giemsa (in Sorenson's buffer) for 10–15 min. All slides were coded, and cells with at least 38 and 21 chromosomes, in mice and Chinese hamsters, respectively, were analyzed for SCEs.

To evaluate replicative index (RI), the frequencies of the first, second, third, and subsequent metaphases were determined in 100 consecutive metaphase cells from each of the four cultures. Those cells whose DNA had replicated exclusively before the addition of BrdU could not be distinguished from cells at first metaphase, and those that had gone through three or more cell cycles were included as third mitoses. The RI was calculated as follows: $RI = (1M_1 + 2M_2 + 3M_3)/100$, where M_1, M_2, and M_3 represent percentages of first, second, and third cycle metaphases, respectively [Schneider and Lewis, 1981].

To relate the SCE frequency per cell to the relative amounts of DNA content in mouse and Chinese hamster, SCEs per pg DNA were calculated by using values of 12.8 and 12.6 pg DNA per cell for mouse and Chinese hamster, respectively [Bachmann, 1972; Uggla and Natarajan, 1982].

Statistical Analysis

The data were arranged as a nested factorial experiment with animal nested within species and SCEs (ie, baseline or induced) and analyzed as a four-way analysis of variance. Because the three-way interaction for species, SCE, and cell type turned out to be significant, each variable was further analyzed separately for each combination of the other two variables. This was treated as nested data for species and SCE, with animal nested within each of these variables, and as blocked data for cell type with animal forming the blocks. The RI data from treatment and control animals were analyzed using analysis of variance procedure.

RESULTS

The data on baseline SCE levels in bone marrow and spleen cells of mice and Chinese hamsters are presented in Table I. The data represent two separate experiments with two animals in each experiment. Both experiments were performed under identical conditions. On the average, the SCE levels per cell in bone marrow and spleen, respectively, were 7.80 and 7.65 for mice and 7.30 and 6.69 for Chinese hamsters. Statistical significance levels of these data are presented in Table II. The baseline SCE levels on per cell as well as on per pg DNA basis did not differ significantly between cell types in mice and between species in bone marrow. However, SCE levels differed significantly between species in spleen ($P \leqslant .01$) and between cell types in Chinese hamsters ($P \leqslant .05$). When the data were analyzed for SCE per chromosome rather than per cell, the two species were significantly different ($P \leqslant .01$). The SCE frequency per chromosome was almost 66% higher in both

TABLE I. Baseline Sister Chromatid Exchanges in Bone Marrow and Spleen Cells of Mouse and Chinese Hamster*

Animal culture	Mouse				Chinese hamster			
	SCEs/Cell ± SE	SCEs/pg DNA[a]	SCEs/Chromosome	RI	SCEs/Cell ± SE	SCEs/pg DNA	SCEs/Chromosome	RI
Bone marrow								
1	7.88 ± 0.47	0.616	0.199	2.04	6.52 ± 0.42	0.517	0.298	1.94
2	7.88 ± 0.46	0.616	0.198	2.00	8.24 ± 0.44	0.654	0.377	2.04
3	7.80 ± 0.50	0.609	0.196	2.21	7.08 ± 0.44	0.562	0.325	1.91
4	7.64 ± 0.48	0.597	0.192	2.05	7.36 ± 0.47	0.584	0.336	2.03
Mean	7.80 ± 0.07	0.609	0.196	2.08	7.30 ± 0.41	0.579	0.334	1.98
Spleen								
1	7.60 ± 0.37	0.594	0.191	1.98	6.72 ± 0.37	0.533	0.309	2.02
2	7.68 ± 0.42	0.600	0.193	2.06	6.64 ± 0.41	0.527	0.305	1.89
3	7.60 ± 0.43	0.594	0.192	1.93	6.56 ± 0.50	0.521	0.301	2.10
4	7.72 ± 0.38	0.603	0.194	2.02	6.84 ± 0.40	0.543	0.314	1.94
Mean	7.65 ± 0.03	0.598	0.193	2.00	6.69 ± 0.07	0.531	0.307	1.99

*Twenty-five second division cells for SCE and 100 metaphase cells for RI were scored from each animal culture.
[a]Data derived from Uggla and Natarajan [1982].

TABLE II. Statistical Significance Levels of Sister Chromatid Exchanges Between Different Variables Analyzed†

	Mouse versus Chinese hamster				Bone marrow versus spleen				Baseline versus induced[a]			
	Baseline		Induced		Baseline		Induced		Mouse		Chinese hamster	
Basis	Bone marrow	Spleen	Bone marrow	Spleen	Mouse	Chinese hamster	Mouse	Chinese hamster	Bone marrow	Spleen	Bone marrow	Spleen
Cell	NS	**	**	**[b]	NS	*	**	**	**	**	**	**
pg DNA	NS	**	**	**[b]	NS	*	**	**	**	**	**	**
Chromosome	**	**	**	**	NS	NS[c]	**	**	**	**	**	**

†Statistical significance levels: NS, not significant; *, $P \leq .05$; **, $P \leq .01$.
[a]10 and 20 mg cyclophosphamide/kg body weight.
[b]$P \leq .05$ for 20 mg cyclophosphamide/kg body weight.
[c]Statistical significance discrepancy due to variability in the number of chromosomes/cell scored.

spleen and bone marrow cells of Chinese hamsters than in those of mice. This was consistent in both experiments.

Treatment of animals with cyclophosphamide yielded a dose-related SCE increase in both mouse and Chinese hamster cells. SCEs caused by cyclophosphamide (10 mg/kg, Table III) differed significantly over controls in both species and in both cell types when the data was expressed on per cell, per pg DNA, and per chromosome basis ($P \leqslant .01$). The spleen cells were more sensitive than bone marrow cells in both species and differed significantly ($P \leqslant 0.01$). In general, the first, second, third and subsequent cell divisions were in close approximation in the study, and thus, the RI data did not differ significantly between treatments and controls.

The data on cyclophosphamide (20 mg/kg)-induced SCEs in bone marrow and spleen cells are shown in Table IV. With 6 hr posttreatment of animals, cyclophosphamide induced about three and two times more SCEs than the baseline levels, respectively, in mouse and Chinese hamster bone marrow cells on a per cells basis. The differences between baseline and induced SCE frequencies and between induced SCE frequencies of mice and Chinese hamsters were statistically significant ($P \leqslant .01$). This statistical difference was also noticed when the data were expressed on SCE per pg DNA content. Even though the mouse bone marrow cells had almost 33% higher SCEs per cell than Chinese hamster, the Chinese hamsters showed about 22% higher SCEs per chromosome than mice. The differences for both per cell and per chromosome were statistically significant ($P \leqslant .01$). The results on spleen cells indicated nearly a four-fold increase in both species over controls and differed significantly ($P \leqslant .01$). Although the SCEs per cell and per pg DNA did not vary greatly between species, the difference between species was significant ($P \leqslant .05$). The same data when analyzed on per chromosome basis, however, were statistically significant between species and cell types at a higher significance level ($P \leqslant .01$). Generally, the RI was about the same in both species and cell types following animal treatment with cyclophosphamide (20 mg/kg) in comparison with respective controls.

DISCUSSION

It is known that BrdU induces SCEs and that baseline SCE levels are dependent on the concentration of BrdU [Carrano et al, 1980]. In the present study with the same concentration of BrdU (20 μM), the control level of SCEs per cell and per pg DNA remained about the same in both cell types in mouse and in bone marrow cells for mouse and Chinese hamster. A similar control SCE frequency level has been reported in Fisher 344 rat bone marrow cells in vitro [Dearfield et al, 1984]. Also, nonsignificant differences of baseline SCE frequencies have been reported by Conner et al [1979] in in vivo mouse bone marrow, alveolar macrophage, and liver cells. In the present study, however, the Chinese hamster spleen cells had significantly lower SCE levels than bone marrow cells, and these levels were also lower than mouse spleen cells. This could be accounted for by a different metabolism of BrdU. A slightly lower baseline SCEs in Sprague-Dawley rat bone marrow and higher baseline SCEs in rat and mouse lymphocytes than those observed in this study have been reported indicating tissue, strain, and species variations and specificities [Dearfield et al, 1984; Takeshita and Conner, 1984]. It may be observed that the Chinese hamster chromosomes ($2n = 22$) showed higher incidence of SCEs per chromosome than mouse chromosomes ($2n = 40$). Since the amount of DNA and the number of SCEs

TABLE III. Cyclophosphamide (10 mg/kg)-Induced Sister Chromatid Exchanges in Bone Marrow and Spleen Cells of Mouse and Chinese Hamster[†]

Animal culture	Mouse				Chinese hamster			
	SCEs/cell ± SE	SCEs/pg DNA[a]	SCEs/Chromosome	RI	SCEs/cell ± SE	SCEs/pg DNA	SCEs/Chromosome	RI
Bone marrow								
1	12.68 ± 0.59	0.991	0.318	1.98	10.24 ± 0.48	0.813	0.468	1.94
2	13.48 ± 0.50	1.053	0.338	1.89	10.00 ± 0.43	0.794	0.458	2.04
3	12.12 ± 0.51	0.947	0.304	1.91	10.92 ± 0.50	0.867	0.499	2.00
4	13.04 ± 0.61	1.019	0.327	2.10	11.04 ± 0.56	0.876	0.506	2.05
Mean	12.83 ± 0.33	1.002	0.322	1.97	10.55 ± 0.29	0.837	0.483	2.01
Spleen								
1	14.72 ± 0.58	1.150	0.370	1.85	19.72 ± 0.55	1.565	0.903	2.09
2	15.16 ± 0.65	1.184	0.381	1.95	18.88 ± 0.61	1.498	0.863	1.91
3	15.36 ± 0.62	1.200	0.386	2.09	18.20 ± 0.56	1.444	0.830	2.01
4	13.88 ± 0.53	1.084	0.348	1.93	17.72 ± 0.61	1.406	0.810	1.99
Mean	14.78 ± 0.38	1.155	0.371	1.96	18.63 ± 0.50	1.479	0.852	2.00

[†]Baseline SCEs (Table I) have not been subtracted. Twenty-five second division cells for SCE and 100 metaphase cells for RI were scored from each animal culture.

[a]Data derived from Uggla and Natarajan [1982].

TABLE IV. Cyclophosphamide (20 mg/kg)-Induced Sister Chromatid Exchanges in Bone Marrow and Spleen Cells of Mouse and Chinese Hamster[†]

Animal culture	Mouse				Chinese hamster			
	SCEs/Cell ± SE	SCEs/pg DNA[a]	SCEs/Chromosome	RI	SCEs/cell ± SE	SCEs/pg DNA	SCEs/Chromosome	RI
Bone marrow								
1	22.68 ± 1.01	1.772	0.571	1.85	13.72 ± 0.62	1.089	0.628	2.00
2	21.88 ± 1.00	1.709	0.549	1.91	14.48 ± 0.74	1.149	0.665	2.09
3	19.76 ± 0.76	1.544	0.498	1.95	15.52 ± 0.69	1.232	0.712	1.97
4	20.44 ± 0.76	1.597	0.514	2.01	13.24 ± 0.76	1.051	0.606	1.93
Mean	21.19 ± 0.77	1.656	0.533	1.93	14.24 ± 0.57	1.130	0.653	2.00
Spleen								
1	27.24 ± 1.26	2.128	0.688	1.95	29.04 ± 1.28	2.305	1.331	2.01
2	27.00 ± 1.51	2.109	0.679	2.06	29.52 ± 1.40	2.343	1.354	2.01
3	27.88 ± 1.39	2.178	0.702	1.93	27.40 ± 1.75	2.175	1.254	2.03
4	27.20 ± 1.43	2.125	0.686	1.99	28.92 ± 1.49	2.295	1.323	2.00
Mean	27.33 ± 0.22	2.135	0.689	1.98	28.72 ± 0.53	2.279	1.316	2.01

[†]Baseline SCEs (Table I) have not been subtracted. Twenty-five second division cells for SCE and 100 metaphase cells for RI were scored from each animal culture.

[a]Data derived from Uggla and Natarajan [1982].

per cell are approximately the same in two species, it would appear that the number of baseline SCEs within a cell have been distributed proportionately among the chromosomes. It should be noted that a comparison of SCEs per chromosome is meaningful when made between cell types or experiments within a species. For interspecies comparison, it appears that SCEs per pg DNA as reported by Uggla and Natarajan [1982], would be more meaningful.

Cyclophosphamide requires metabolic activation for its mutagenicity and clastogenicity. The rapid increase in SCE response in mice and Chinese hamsters by acute exposure to cyclophosphamide in this study parallels the reported rapid production of active metabolites following cyclophosphamide administration in rats [Natarajan et al, 1983]. Similar increase in SCE frequencies after cyclophosphamide treatment has been reported in rat lymphocytes and bone marrow [Dearfield et al, 1984], in mouse lymphocytes, [Takeshita and Conner, 1984; Wilmer et al, 1984], and in Chinese hamster bone marrow cells in vivo [Neal and Probst, 1983].

At the same concentrations (10 and 20 mg/kg), cyclophosphamide caused relatively higher SCEs per pg DNA in the bone marrow of mice than in Chinese hamsters. Similar differential sensitivity of bone marrow cells of mice and Chinese hamsters, as measured by chromosomal aberrations and micronuclei following an intraperitoneal injection of cyclophosphamide (40 mg/kg), was reported by Goetz et al [1975]. Spleen cells in both species, however, are more sensitive than bone marrow cells ($P \leqslant .01$). Spleen is the largest accumulation of lymphatic tissue in animals and is the site of maturation, removal, and storage of lymphocytes, which later pass into the blood. Cyclophosphamide has been shown to exert toxic effects on mammalian hematopoietic tissues. This toxicity is manifested as leukopenia [De Wys et al, 1970; Dumont, 1974; Luster et al, 1981; Mansour and Nelson, 1977; Turk and Poulter, 1972], destruction of lymphocyte germinal centers in spleen and lymph nodes [Stockman et al, 1973], and cytotoxic effects on hemic precursors [Wierda and Pazdernick, 1979]. Recently, Wilmer et al [1984] reported a relatively higher sensitivity of B-lymphocytes than T-lymphocytes in in vivo exposure of mice to cyclophosphamide followed by blood culture. In the present study, even though 2-mercaptoethanol, a nonspecific mitogen, was used to stimulate spleen cells in addition to lipopolysaccharide, a B-lymphocyte mitogen, the results parallelled those of Wilmer et al [1984] who used only lipopolysaccharide. It is also possible that different metabolic patterns of cyclophosphamide in bone marrow and spleen of mice and Chinese hamsters may account for the observed differences in SCE frequencies. However, the actual enzyme activities responsible for such differences have not yet been identified. It may be noted that interspecies and intertissue/cell variations are determined not only by a karyologic characteristic of an individual species, but also by a pharmacokinetic drug effect in the organism and subsequently in the culture. The data on cell cycle kinetics indicated that cyclophosphamide, at the concentrations of 10 and 20 mg/kg, was not toxic to Chinese hamsters and mice and did not cause cell cycle delay in culture.

The reported in vivo exposure and in vitro culture SCE assays in bone marrow and spleen cells of mouse and Chinese hamster are particularly useful as sensitive tools for the detection of genotoxic agents, especially indirect-acting mutagens/clastogens and potential carcinogens. These assays may be comparable to those of human in vivo cytogenetic monitoring assays and may be useful for comparing the results. In addition, bone marrow and spleen are two important tissue/cell types that have been used for in vivo SCE studies in animals. Thus, these assays may become useful tools in the area of comparative mammalian cytogenetics.

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