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CYCLOPHOSPHAMIDE-INDUCED *IN VIVO* SISTER CHROMATID EXCHANGES (SCE) IN *MUS MUSCULUS*. III. QUANTITATIVE GENETIC ANALYSIS

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ABSTRACT

In vivo cyclophosphamide (CP)-induced sister chromatid exchanges (SCEs) were evaluated in females from five genetic strains of mice (C57BL/6J, C3H/S, 129/ReJ, BALB/c and DBA/2) and their F_1 hybrids. Baseline (noninduced) SCE values differ significantly among strains, 129/ReJ having the lowest and DBA/2 having the highest mean SCE per cell values. In general, the baseline SCE of a given F_1 is within the range of its corresponding parental strains or near the lower parental value. Furthermore, there is a genotype-dependent increase in mean SCEs per cell with CP dose. Strain differences in SCE induction are noted particularly at the two higher CP doses (4.50 and 45.0 mg/kg). In general, F_1 hybrids involving a strain with high induced SCEs and a strain with low induced SCEs exhibit mean SCE values that are closer to the value of the lower strain. F_1s involving two strains with high SCEs or two strains with low SCEs yield SCEs not different from parental strains. The method of diallel cross analysis showed the order of dominance of these strains in SCE induction to be 129/ReJ BALB/c C3H/S DBA/2 C57BL/6J. These results support the involvement of predominantly nonadditive genetic factors as major gene(s) in SCE induction. In addition, involvement of random and independent events in SCE induction is suggested by the distribution of SCEs which follows a Poisson distribution.

SISTER chromatid exchanges (SCEs) represent the interchange of DNA between replication products at apparently homologous loci (LATT *et al.* 1979). They presumably require DNA breakage and reunion (LATT 1981), and the exchanges take place between double DNA strands (KATO 1977) having the same polarity (LATT and SCHRECK 1980). However, the molecular mechanism of SCE formation and its biological significance remain obscure. In spite of these uncertainties, this cytological phenomenon is considered to be a rapid and sensitive method to detect the effect of mutagens with few false-positive results *in vivo* (STETKA and WOLFF 1976a; NAKANISHI and SCHNEIDER 1979) and *in vitro* (STETKA and WOLFF 1976b; MADLE and OBE 1977), particularly in mammals. It has been extremely useful in differentiating among chromosome fragility diseases (LATT *et al.* 1980), providing information about the structure of the eukaryotic chromosome (HSU and PATHAK 1976; REIMER and SINGH 1982) and is helping to elucidate the phenomenon of aging at the

cellular level (KRAM *et al.* 1978; SCHNEIDER *et al.* 1979; SCHNEIDER and GILMAN 1979; NAKANISHI, DEIN and SCHNEIDER 1980; REIMER and SINGH 1983).

It is natural to think of a genetic basis for the observations on SCE formation. In fact, a number of features from reported results strongly suggest genetic contributors to the phenomenon of SCE formation. These include mutagen specificity (LATT *et al.* 1981), species specificity (WOLFF and RODIN 1978; BARNETT and WALLACE 1982), the effect of genetically determined chromosome fragility disorders (LATT *et al.* 1980), cell type differences (MITCHELL, MEHER-HOMJI and BAKER 1982), strain differences *in vivo* (BIEGEL, BOGGS and CONNER 1980; GALLOWAY *et al.* 1980; DRAGANI, ZUNINO and SOZZI 1981; REIMER and SINGH 1982) and strain-dependent aging response (REIMER and SINGH 1983). Most of these generalities have been observed *in vitro* and *in vivo* in response to a number of chemicals and mutagens, some of which [*e.g.*, cyclophosphamide (CP)] require metabolic activation prior to becoming effective SCE inducers (HILL 1975). The genetic determinants for SCE formation may affect genetically mediated metabolic activation or deactivation of the inducer (MITCHELL, MEHER-HOMJI and BAKER 1982), genetically determined enzymes providing protection against breaks [*e.g.*, superoxide dismutase, catalase, etc. (MORGAN, CONE and ELGERT 1976; SPEIT, VOGEL and WULF 1982)] and/or genetically influenced rate of DNA replication and repair (SHIRAISHI, MINOWADA and SANDBERG 1979). It is realized (LATT *et al.* 1981) that appropriate studies on strains of mice or other rodents will be desirable in evaluating the role and nature of possible genetic determinants of SCE formation. These *in vivo* studies, using 5-bromo-2′-deoxyuridine (BrdU) administration, permit analyses that are complementary to *in vitro* trials and provide information which is otherwise unobtainable. We evaluated differences among genetic strains of mice in SCE formation *in vivo* in response to CP. This metabolically activated alkylating agent induces high levels of SCEs with minimal chromosomal aberrations (NAKANISHI and SCHNEIDER 1979). Here, we report and discuss our results on CP-induced SCEs in five strains of mice and their F_1 hybrids. The results are discussed in the light of genetic determinants involved in the mechanism and rate of SCE formation.

MATERIALS AND METHODS

Five genetic strains of mice: C57BL/6J, 129/ReJ (The Jackson Laboratory, Bar Harbor, Maine), C3H/S, BALB/c and DBA/2 (Canadian Breeding Farm, Charles River, Quebec) were used in this study. Mice were maintained in the animal care facilities of the University of Western Ontario, London, Canada, under standard conditions. They were paired in breeding cages to produce all possible F_1s and genotypes representing each of the five strains. Female mice (11.28 ± 0.95 wk old) representig ten hybrids and five parental strains were treated with 9-hourly intraperitoneal (ip) injections of BrdU (~214 mg/kg/hr) following the method of REIMER and SINGH (1982). This treatment was found to be satisfactory to differentially label metaphase chromosomes of the bone marrow cells. A single ip injection of 0, 0.045, 0.45, 4.50 or 45.0 mg/kg of CP was administered 15 min after the last BrdU injection. Twelve hours later, a single ip injection of ~4 mg/kg of colcemid was given to each animal. Mice were sacrificed 2 hr later by cervical dislocation. The femoral bone marrow cells were collected. Slide preparation and fluorescence plus Giemsa staining followed the procedure of PERRY and WOLFF (1974). BrdU (Sigma), CP (Sigma), colcemid (Gibco) and other chemicals used were of analytical grade. All solutions were stored in light tight containers at 5° and used within 2 weeks.

Total SCEs per cell in 20 well-spread complete second-replication metaphase cells were scored for each animal. A number of CP treatment and genotype combinations were repeated on two/three animals; others represent values on a single individual. Analysis of variance was used to evaluate the significance of differences in SCE frequencies between individuals within a CP dose and genotype combination, between genotypes and between CP doses. Further analysis followed statistical comparisons of means and variances of appropriate combinations. The method of diallel cross analysis (HAYMAN 1954) was used to determine genetic components of variation in the induction of SCEs.

RESULTS

Table 1 shows the SCE frequencies (mean ± S.E.) for different doses of CP in five inbred strains of mice (diagonal) and their F_1 hybrids. These values are considered representative for each genotype and CP dose combination, since differences between individuals (where replictions are available) within a given combination are not significant (Table 2). At a high CP dose (45.0 mg/kg), the number of well-spread metaphases with differentially labeled chromatids was limited. This treatment resulted in extensive chromosome breakage and cytotoxicity. SCE values for this treatment, therefore, are sometimes based on fewer than 20 cells per animal. The data set presented in Table 1 permits evaluation of genotype differences in the baseline SCEs and the CP dose response. The baseline (CP = 0 mg/kg) SCE values differ significantly ($P < 0.001$) among genotypes. Among the strains, the 129/ReJ had the lowest (8.98 ± 0.68) and DBA/2 the highest mean number (14.50 ± 1.01) of SCEs per cell. In general, the baseline SCE value of a given F_1 is within the range of its corresponding parental strains or near the lower parental value. There is a significant genotype-dependent increase in mean SCEs per cell with CP dose ($P < 0.001$). The dose-response curves (SCEs plotted against the square root of CP dosage—not given), although similar in shape (reflecting a linear relationship), differ significantly among genotypes; strains 129/ReJ, DBA/2 and BALB/c have smaller increases with CP dose [estimates of m (rate of increase] being 5.44, 5.81, and 6.44) as compared with C3H/s and C57BL/6J (with m = 7.09 and 8.29, respectively).This linear relationship of SCE with the square root of CP dose may represent the involvement of a one-hit phenomenon of DNA damage caused by CP metabolites. The strain differences in CP-induced SCEs per cell are also reflected in the difference in SCE values between CP doses 0 and 4.5 mg/kg (for 129/ReJ and DBA/2, this difference is 37 and 38, respectively, and for C3H/S and C57BL/6J it is 50 and 55, respectively).

The strain differences in SCEs per cell were highly significant ($P < 0.01$) at the two higher doses of CP (4.50 and 45.0 mg/kg). Additional genetic analysis of induced SCEs at the 4.50-mg/kg CP dose was attempted in which the number of metaphase cells with good differential staining was found to be adequate. In general, F_1s involving a strain with high induced SCEs and a strain with low induced SCEs exhibit mean SCE values closer to the lower parent, particularly at the two higher CP doses (*e.g.*, C57BL/6J × 129/ReJ). Also F_1s involving two strains with high SCEs or two strains with low SCEs yield mean SCEs not different from the parental values at most CP treatments. The exception to this pattern is the cross BALB/c × 129/ReJ, which yielded

TABLE 1

SCEs per cell (means ± S.E.) in five inbred strains of mice and their F_1 hybrids at different doses of CP

	CP dose (mg/kg)	C57BL/6J	C3H/S	BALB/c	129/ReJ	DBA/2
C57BL/6J	0.000	11.54 ± 0.59[a]		10.13 ± 0.69[a]	12.01 ± 0.72[a]	9.40 ± 0.81
	0.045	12.90 ± 1.38	13.75 ± 1.97	9.45 ± 1.20	7.95 ± 0.72	15.60 ± 1.60
	0.450	14.70 ± 1.49	14.50 ± 1.30	12.75 ± 1.78	8.95 ± 0.98	15.70 ± 2.40
	4.500	25.39 ± 1.03[a]	20.80 ± 2.06	19.40 ± 2.33	16.67 ± 0.93[a]	27.70 ± 1.91
	45.000	66.50 ± 4.30[d]	56.45 ± 1.67	54.70 ± 2.50[d]	49.30 ± 3.60[d]	50.00 ± 3.70[d]
	m^e	8.29	6.79	6.67	6.15	5.72
C3H/S	0.000		13.30 ± 1.64	8.15 ± 0.55	11.55 ± 1.19	14.00 ± 1.50
	0.045		17.95 ± 1.80	14.00 ± 2.59	13.60 ± 1.40	11.95 ± 1.07
	0.450		21.40 ± 1.52	16.50 ± 2.11	13.30 ± 1.30	13.95 ± 1.08
	4.500		27.45 ± 2.07	22.75 ± 1.95	18.50 ± 1.30	16.10 ± 1.05
	45.000		63.10 ± 3.60[d]	60.10 ± 4.40[d]	49.80 ± 2.90[d]	47.80 ± 3.60[d]
	m^e		7.09	7.37	5.71	5.29
BALB/c	0.000			10.30 ± 0.78[a]	8.98 ± 0.68[a]	11.45 ± 0.85
	0.045			11.05 ± 1.74	11.40 ± 1.13	10.15 ± 0.86
	0.450			12.15 ± 1.39	11.00 ± 0.90	12.00 ± 1.18
	4.500			22.20 ± 0.85[a]	16.27 ± 0.89[a]	21.55 ± 1.29
	45.000			52.70 ± 2.90[d]	28.08 ± 1.36[c]	49.00 ± 3.40[d]
	m^e			6.44	2.63	5.86
9/ReJ	0.000				8.98 ± 0.68[a]	11.93 ± 1.24[b]
	0.045				9.95 ± 1.05	14.80 ± 0.99[b]
	0.450				15.20 ± 1.88	13.80 ± 1.42
	4.500				16.27 ± 0.89[a]	16.65 ± 1.53
	45.000				46.40 ± 3.70[d]	60.40 ± 3.69[d]
	m^e				5.45	7.21
DBA/2	0.000					14.50 ± 1.01
	0.045					13.20 ± 1.18
	0.450					13.75 ± 1.22
	4.500					24.55 ± 1.56
	45.000					51.40 ± 6.70
	m^e					5.81

Based on [a]three replications, 20 cells/animal; [b] and [c]two replications, 20 and 10 cells/animal, respectively; [d]10 to 13 cells from a single animal; others on 20 cells on a single individual.
[e]m = rate of increase in SCEs with increasing doses ($X^{1/2}$) of CP.

TABLE 2

Individual variations associated with SCEs per cell in bone marrow cells of mice in vivo

Genotype	CP dose (mg/kg)	Mean SCE/cell/animal[a] 1	2	3	F	P[b]
BALB/c	0	8.80	10.40	11.75	0.042	0.041
	4.5	22.25	23.50	21.50	0.001	0.001
C57BL/6J	0	11.6	11.2	11.85	0.003	0.004
	4.5	23.3	28.7	23.85	0.103	0.097
129/ReJ	0	9.55	9.55	7.85	0.024	0.024
	4.5	19.45	14.35	15.00	0.124	0.116
129/ReJ × C57BL/6J	0	11.55	12.90	10.70	0.029	0.029
	4.5	18.85	18.25	17.90	0.026	0.026
129/ReJ × BALB/c	0	10.50	10.75	10.63	0.000	0.006
	4.5	15.95	18.55	17.45	0.035	0.034
C57BL/6J × BALB/c	0	9.90	11.40	9.10	0.033	0.033
	4.5	20.85	19.40	20.50	0.000	0.005

[a] Based on 20 or more cells in metaphase per animal.
[b] Probability that intraindividual differences are significant.

drastically reduced SCEs at the highest CP dose (45.0 mg/kg) (28.08 as compared with 46.40 and 52.70 parental values in repeated experiments).

The CP dose-dependent increase in the mean SCE values is also associated with an increase in the variance of SCEs per cell in most genotypes (see S.E. values in Table 1). For comparison purposes, we present the distribution of SCEs per cell in the two strains with high (C57BL/6J) and low (129/ReJ) SCE induction and their F_1 hybrid, in response to different CP doses, in Figure 1. It shows that the number of cells with higher SCEs and the range of SCEs per cell are relatively higher in C57BL/6J than in 129/ReJ, particularly at the higher CP doses (4.50 and 45.0 mg/kg). Also, the F_1 hybrids reflect SCE distributions similar to 129/ReJ that are significantly different from C57BL/6J ($P < 0.05$). This is also true for the mean values represented in the inset of this figure. A similar comparison involving two strains with relatively low SCE induction (DBA/2 and BALB/c) and their F_1 hybrid results in SCE distributions similar to the parents. The same could be said about strains with high SCE inductions and their F_1 hybrid. All of these distributions, however, follow a Poisson distribution with most cells having smaller number of SCEs in a given genotype CP dose combination.

The data presented in Table 1 on five genetic strains and all of their possible F_1s (excluding reciprocals) permit the evaluation of the involvement of genetic parameters in SCE induction. This was attempted for observed SCE values at the 4.50-mg/kg dose of CP (Table 1) where adequate differentially labeled metaphase cells were available, and genotype differences were found to be significant ($P < 0.05$); 129/ReJ > BALB/c > DBA/2 = C57BL/6J = C3H/

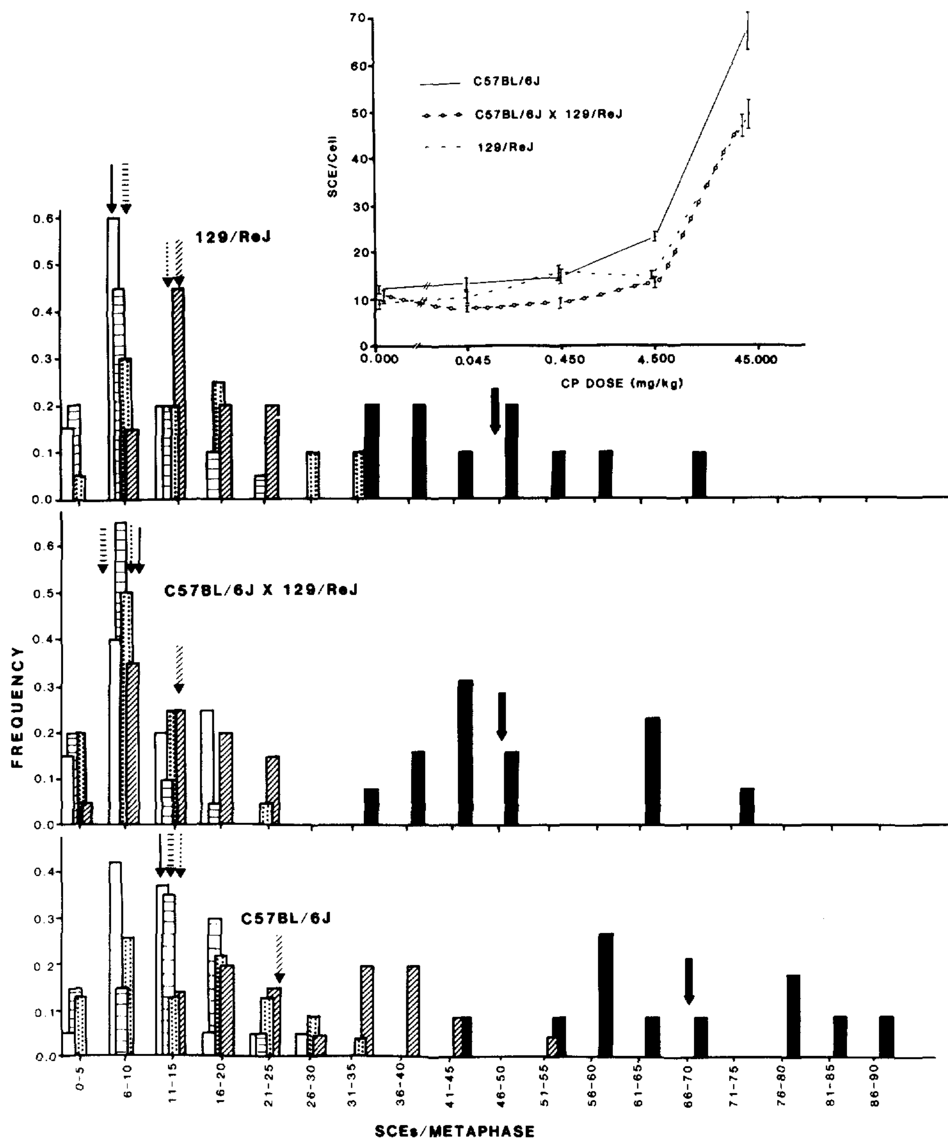

FIGURE 1.—Distribution of SCEs per cell in C57BL/6J, 129/ReJ and their F_1 hybrid (C57BL/6J × 129/ReJ) at five CP doses: □, 0 mg/kg; ▤, 0.045 mg/kg; ▨, 0.450 mg/kg; ▧, 4.50 mg/kg; ■, 45.0 mg/kg. Arrows indicate mean SCE values at the appropriate CP dose. Inset shows SCEs per cell (mean ± S.E.) as a function of CP dose.

S. A simple comparison of the ten F_1 values with their corresponding parental strains indicates that most F_1 values are either not different from or lower than the lower parent, a phenomenon expected from allelic and nonallelic interactions rather than from the additive action of genes. This data set also permits the use of the method of diallel cross (partial) analysis, normally applied to quantitative observations (FALCONER 1981). Such an analysis (HAYMAN 1954) yielded a (Vr, Wr) graph (Figure 2) as well as estimates on different compo-

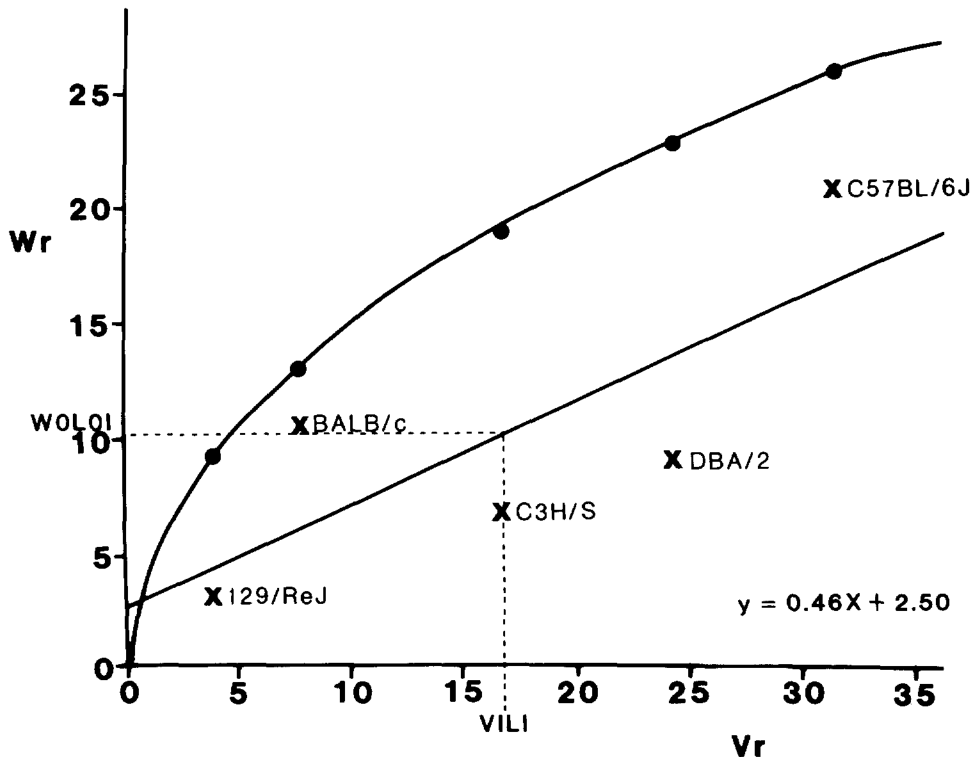

FIGURE 2.—(Vr, Wr) graph of observed mean SCE values at 4.5 mg/kg of CP based on five strains of mice and their F_1 hybrids.

nents of the genetic determinants. The graph shows that the order of dominance of these strains is 129/ReJ > BALB/c > C3H/S > DBA/2 > C57BL/6J. This dominance relationship should be interpreted with caution as mean SCEs for C3H/S, DBA/2 and C57BL/6J are not significantly different from each other. However, these observations suggest that the relatively low SCE induction property is dominant over high SCE induction. These conclusions are also supported by the estimates of different genetic components of variation. Here, the estimate $(H_1/D)^{1/2} = 1.43$, which measures the mean degree of dominance over all loci, suggests overdominance. The estimate $(H_2/4H_1) = 0.26$ agrees with the observation that H_1 is not significantly different from H_2, suggesting that positive and negative genetic contributors are distributed in similar proportions in these parental strains. Also $h^2/H_2 = 0.87$ suggests that at least one gene controlling SCE formation exhibits some degree of dominance, and, finally, the term $[((4DH_1)^{1/2} + F)/((4DH_1)^{1/2} - F)] = 0.85$, which is near unity, implies equality between the numbers of dominant and recessive alleles in the parental strains.

The conclusion that SCE induction is transmitted with a genic basis could be drawn from the rate of SCE increase with CP dose in different genotypes. We obtained the rate of increase in SCEs for each genotype in a regression equation ($y = m\sqrt{x} + b$) with increasing dose (x) of CP, where m and b represent

the slope and the intercept of the regression line, respectively. The rate estimates (m) of five strains and their F_1's (without reciprocals) are also given in Table 1. It shows that m varies among strains (C57BL/6J = 8.29, C3H/S = 7.09, BALB/c = 6.44, 129/ReJ = 5.45 and DBA/2 = 5.81). The F_1s with C57BL/6J as one of the parents yield m values between 5.72 (DBA/2 × C57BL/6J) and 6.79 (C3H/S × C57BL/6J), which are different from C57BL/6J and closer to the other parent with a lower m. Other F_1's also follow this trend and have m values closer to the parent with the lower m value. The exception to this pattern is 129/ReJ × DBA/2 with a significantly higher m value (7.21) and 129/ReJ × BALB/c with a significantly lower m value (2.63), in repeated trials, than the corresponding parental values.

A diallel cross analysis on m values of these genotypes also suggests that possible genetic contributors of this feature involve a high degree of dominance $[(H_1/D)^{1/2} = 3.1]$, the positive and negative contributors being evenly distributed among strains $[(H_2/4H_1) = 0.25]$. These genetic estimates, however, should be interpreted with some caution, since they involve a diallel cross analysis on an estimate (slope = m) rather than an actual phenotypic observation on a set of strains and their F_1 hybrids.

DISCUSSION

Assays for SCEs are among the most common tests of DNA damage by chemicals and other mutagens in mammalian systems *in vivo* (STETKA and WOLFF 1976a; NAKANISHI and SCHNEIDER 1979) and *in vitro* (STETKA and WOLFF 1976b; MADLE and OBE 1977). They are generally evaluated after BrdU incorporation during mitotic replication, followed by exposure to the chemical or mutagen under investigation on a given cell line. Although a number of cell lines from a given individual is recommended for a realistic evaluation of the SCE level following exposure (CONNER, BOGGS and TURNER 1978), little attention is paid to the genetic background of the individual or cell line, with the exception of "chromosomal breakage diseases." A number of recent reports have suggested that genetic variation is associated with SCE frequencies among families (COHEN *et al.* 1982), strains (BIEGEL, BOGGS and CONNER 1980; DRAGANI, ZUNINO and SOZZI 1981; REIMER and SINGH 1982) and age (SCHNEIDER *et al.* 1979; REIMER and SINGH 1983) but to our knowledge no comprehensive attempt has been made to understand the nature of these genetic contributors. The genetic differences may be involved in providing protection against breaks and/or affecting the repair process, two of the realized components of the phenomenon of SCE formation (LATT *et al.* 1980; SPEIT and VOGEL 1982). Furthermore, if an inducer needs to be metabolized before it becomes effective, genetic factors may be of significance.

In particular this study suggests the involvement of genetic factors in the formation of SCEs. The baseline SCE values differ among genotypes and the F_1 hybrids show SCE values resembling the parent with the lower counts. There is a dose response with CP that varies among genotypes. In addition, the frequency of cells with smaller numbers of SCEs is higher than the frequency of cells with a larger number of SCEs in each genotype at every dose

of CP (*i.e.*, SCE induction follows a Poisson distribution). The Poisson distribution of induced SCEs suggests that there is random and independent induction of SCEs at least in the bone marrow cells that represent a heterogeneous cell population. The mean and the distribution of SCEs of a given F_1 hybrid follow the pattern of the parental strain with lower mean SCE values, at most CP doses. The mean SCE values of F_1s which are less than the value for either parent may in fact represent complementation or heterosis. A similar pattern is also seen for the rate of SCE induction [analyzed as the slope of the regression line (m)] with CP dose and observed SCEs at a 4.50-mg/kg dose of CP. These two parameters were also analyzed using a common method of quantitative genetic analysis (diallel cross analysis) that substantiates the aforementioned conclusions. It may be pointed out that the results of response at 4.50 mg/kg of CP may apply only to this dose and may not be generalized to the response at any other dose. Furthermore, the two traits analyzed in this report probably have a compound nature and may be genetically independent. Although our results support the involvement of predominantly nonadditive genetic factors in variation in SCE induction liability and may reflect on natural selection for this feature during the evolutionary history of the species (particularly of these strains), our data set does not permit evaluation of the precise role of such genetic determinants in SCE induction. They may be involved in the metabolism of CP, providing protection against breaks and/or the efficiency of DNA repair. Additional studies on enzymes involved in CP metabolism, protection against DNA damage and/or repair may provide insights into the actual role of genetic factors in the formation and susceptibility to induced SCEs. As a word of caution one may suggest the use of experimental animals with more than one genetic background during *in vivo* mutagenicity testing based on SCEs.

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