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Sister chromatid exchange induction and the course of DNA duplication, two mechanisms of sister chromatid exchange induction by ENU and the role of BrdU

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The aims of the present study were to establish the following: (i) the course of sister chromatid exchange (SCE) induction by ethylnitrosourea (ENU) in the first, second and third divisions as a function of the exposure time; (ii) the persistence of SCE-inducing lesions and the determination of whether or not they are always involved in SCE formation; (iii) the effect of bromodeoxyuridine (BrdU) incorporation on the induction and persistence of SCE. Three-way differential staining of sister chromatids in murine bone marrow cells *in vivo* was used in the present study. The results indicate the following: (i) SCE induction in each cell division depends on the course of DNA duplication, suggesting that SCE occurs at the replication fork; (ii) the cell population under study could be considered synchronous and had a cell cycle duration of nearly 9 h; (iii) in the second and third cell divisions ENU preferentially induced SCE in the cycle in which the exposure occurred; (iv) lesions induced by exposure to ENU did not cause SCE at the same site in subsequent divisions; (v) ENU was also capable of producing a long-lasting induction of SCE in BrdU-unsubstituted DNA; (vi) the sensitivity to SCE induction by the mutagen increases nearly proportionally to BrdU incorporation into DNA.

Introduction

Sister chromatid exchange (SCE) is a phenomenon that occurs widely in nature (Chao *et al*., 1985; Escalza *et al*., 1989; Wei *et al*., 1993); this suggests that it represents or is the expression of a biologically relevant process that has been preserved through evolution. However, its biological meaning and the mechanism of formation have not been fully elucidated. Evidence has been obtained that the occurrence of SCE requires the cell to pass through DNA synthesis (Wolff *et al*., 1974); there are also some data suggesting that the replication fork is the site at which this event occurs (Kato, 1980; Cortés *et al*., 1993). It has been reported that SCE could be caused by the presence of DNA lesions at the moment of replication (Kato, 1980) or by the alteration of this latter process (Ishii and Bender, 1980; Nishi *et al*., 1982). DNA synthesis, and particularly formation of the replication fork, is the moment at which SCE most probably occurs, because this is when the homologous double strands are close together and a homologous recombinational event could take place more easily (Morales-Ramírez *et al*., 1990). Clear evidence has recently been reported relating SCE to homologous recombination (Sonoda *et al*., 1999).

In a model of SCE production (Morales-Ramírez *et al*., 1990) based on the recombinational model of Holliday (Dressler and Potter, 1982) and on replicative repair in eukaryotes (Lavin, 1978), a mechanism was proposed that permits DNA duplication in the presence of lesions; these lesions could subsequently be eliminated by an associated repair process or after replication. In such a model, the Holliday intermediate could be resolved by generating a double or simple strand exchange, which determines whether a sister chromatid exchange does or does not occur. In this context, an approach to determining the biological meaning would be to establish whether SCE production is related to the elimination of DNA damage or to lesion tolerance. In order to discriminate between these two possibilities, evidence is required that may allow one to establish whether or not a lesion can cause SCE in subsequent divisions and whether it is common for SCE to occur at the same locus in subsequent cell divisions.

There is evidence that mutagen exposure can induce SCE much later in proliferative cells (Morales-Ramírez *et al*., 1984a), indicating that not all SCE-inducing lesions are repaired during cell division. However, the evidence obtained by the usual protocol of two-tone staining of sister chromatids does not let us clearly determine the persistence or repair of SCE-inducing lesions. Given that differential staining requires two cell divisions, the SCEs analyzed could be the accumulation of SCE occurring in two cell divisions. In order to distinguish SCE induction in each cell division, the mutagen treatment was applied to different groups in each cell cycle; SCE induction in each cell division was estimated by difference (Ockey, 1981; Conner *et al*., 1984; Kaina and Aurich, 1985). However, the cancellation effect (Stetka, 1979) in cells exposed in the first division and the bromodeoxyuridine (BrdU) substitution effect (Ockey, 1981) in those exposed in the second division make interpretation difficult.

A protocol based on the three-way differential staining (TWDS) of SCE (Schvartzman and Goyanes, 1980) allows one to determine SCE occurring in each of three successive cell generations and even the frequency at which a SCE occurs at the same locus. Using this protocol in human lymphocytes or CHO cells *in vitro* it has been reported that a single time exposure to mitomycin C, UV light, ionizing radiation or EMS was capable of inducing SCE in successive divisions, but not at the same locus (Schvartzman *et al*., 1985; Cortés *et al*., 1994). In murine bone marrow cells *in vivo*, also with a single exposure, it was observed that γ-rays caused 100% of lesions capable of inducing SCE at the same locus (Morales-Ramírez *et al*., 1988); likewise, it has been noted that some chemical mutagens produced the same effect, but with 50% probability (Morales-Ramírez *et al*., 1990) or less (Morales-Ramírez *et al*., 1992, 1995).

In the TWDS protocol, the relationship between the time of mutagen exposure and the course of DNA duplication is essential for interpretation, because assuming that SCE occurs at the duplication fork (Kato, 1980; Cortés *et al*., 1993), the

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DNA lesions induced behind the fork would produce SCE during subsequent cell divisions; this could be misinterpreted as a persistence of lesions.

The aims of the present study were as follows: (i) to establish the course of SCE induction in the first (SCE-1), second (SCE-2) and third (SCE-3) divisions depending on the exposure time to ethylnitrosourea (ENU); (ii) to establish if mutagen exposure causes DNA lesions eliciting SCE in successive divisions (persistent) and even at the same locus (tenacious); (iii) to determine the effect of BrdU incorporation into DNA on the induction and persistence of SCE-eliciting lesions.

Materials and methods

Animals

Two- to three-month old BALB/c male mice weighing 30 g were used in these experiments. The animals were housed in plastic cages under controlled conditions of temperature and dark–light periods and were fed with Purina chow for small rodents and water *ad libitum*.

Protocol

The protocol followed to determine SCE induction in three subsequent cell generations in murine bone marrow cells (Morales-Ramírez *et al*., 1988, 1990, 1992, 1995) is based on three-way differential staining of sister chromatids (Schvartzman and Goyanes, 1980). The mice received a low BrdU dose (0.2 mg/kg body wt i.p.) before the first division (time 0) and a high dose (2.0 mg/kg body wt i.p.) before the second cycle of division (12 h after initiation). The BrdU had previously been adsorbed to activated charcoal (Morales-Ramírez, 1980; Morales-Ramírez *et al*., 1984b). A dose of 25 mg/kg ENU (in water) was administered s.c. to different groups of mice 3, 6, 9, 12, 18, 24 or 30 h after the first injection of BrdU (low BrdU dose). Additional experiments were carried out with exposure 3, 6 and 12 h prior to the first BrdU dose. Colchicine (3.75.mg/kg) was administered i.p. 2 h before killing by cervical dislocation and 36 h after the first BrdU injection.

The times of treatment with the two BrdU doses and with colchicine and the differential staining in three tones permitted the selection of cell populations that divided three times between the first administration of BrdU and the end of the experiment, assuming an average generation time of 12 h (Ivett and Tice, 1992). This also allows one to predict the level of BrdU substitution in DNA strands at the moment of ENU administration (Figure 1).

The protocol permits one to score SCEs that occur in the first, second and third divisions in the cells and to infer whether or not they occur at the same site in successive divisions (Figure 1).

Chemicals

BrdU, ENU and colchicine were obtained from Sigma Chemicals.

ENU administration

The ENU dose of 25 mg/kg was established in preliminary experiments using the usual two-tone staining protocol, as the dose that causes the maximum SCE concomitant with only a moderate cytotoxic effect (data not shown). The latter was determined as the effect on the mitotic index and average generation time (Ivett and Tice, 1992).

Slide preparation

The animals were killed by cervical dislocation 2 h after administration of colchicine. Both femurs were dissected and bone marrow cells were obtained by injecting a saline solution at one end of the bone. The cells obtained were treated with a hypotonic solution, fixed as reported previously (Morales-Ramírez *et al*., 1990) and finally dropped onto chilled slides. The slides were dried for at least 24 h before staining by the fluorescence plus Giemsa method (Perry and Wolff, 1974), as slightly modified (Goto *et al*., 1975).

Analysis and statistical methods

The frequencies of SCE-1, SCE-2 and SCE-3 (Figure 1) were scored in 30 cells/animal. SCE-3 in chromosome descendants of the BrdU-unsubstituted DNA strand, which have a dark stained chromatid (SCE-3D), and those of the BrdU-low substituted DNA strand, which have a pale stained chromatid (SCE-3P), were analyzed in the same cells. The statistical significance among groups was determined with Student's *t*-test and the difference in SCE frequency between SCE-3D and SCE-3P was established by the paired *t*-test using the Excel program for PC.

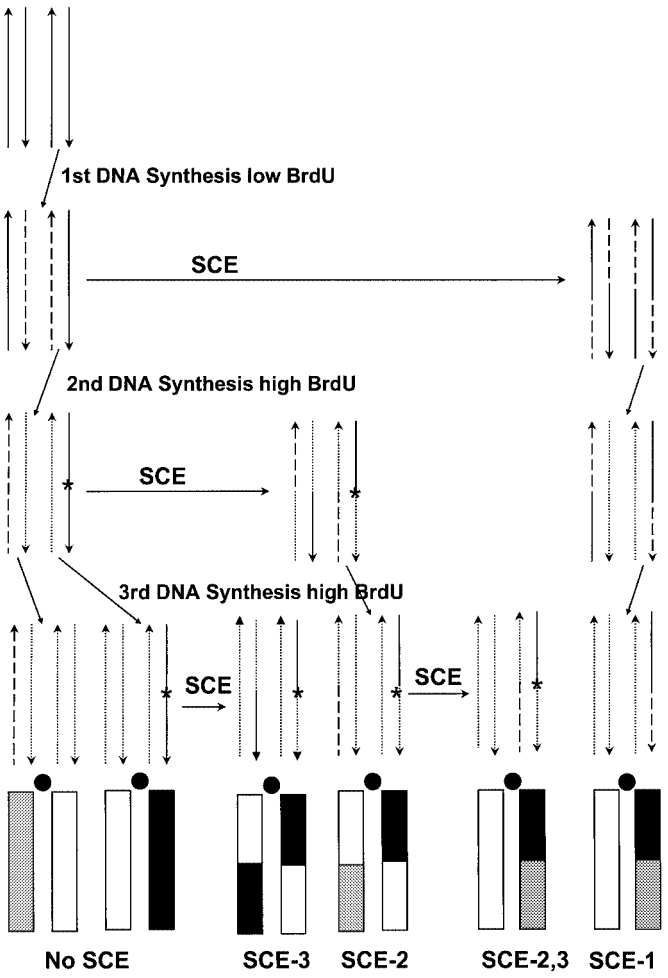

Fig. 1. The TWDS protocol for chromatids is shown. This figure describes how differential BrdU incorporation in three subsequent divisions allows one to determine the SCE in each of the three divisions, as well as to establish the probability that SCE occurs at the same locus in the second and third divisions due to tenacious lesions. As shown, when SCE occurs in these divisions at the same locus, exposure to the mutagen in the second division would paradoxically cause an increase in SCE with the appearance of those occurring in the first division. Continuous lines represent unsubstitution, dashed lines low BrdU substitution and dotted lines high BrdU substitution. The figure shows the dark and pale chromosomes derived from the unsubstituted and BrdU-substituted strands, respectively, present after the first division.

Rationale to achieve the aims

The TWDS protocol for chromatids shown in Figure 1 makes it possible to determine SCE in each of the three subsequent divisions. This allowed us to explore the following.

i. The course of SCE induction in each of the three divisions as these three successive cell divisions progress. This was done by exposing the animals to ENU at different times.
ii. The induction of SCE in successive divisions after exposure (persistence), as well as the probability that SCE occurs at the same locus in the second and third divisions (tenacity). The latter was determined in cells exposed to ENU during the second division (as shown in Figure 1); when SCE occurs in the second and third divisions at the same locus, the exposure in the second division would paradoxically cause an increase in SCEs with the appearance of those occurring in the first division.
iii. The effect of BrdU on SCE induction by ENU. This was done by determining (a) the effect of cumulative incorporation of BrdU in subsequent divisions and (b) the difference in sensitivity to the induction of SCE-eliciting lesions of the BrdU-substituted and BrdU-unsubstituted DNA strands. Such a difference was established by comparing the induced frequencies of SCE-3D with those of SCE-3P.

Results

Course of SCE-1, SCE-2 and SCE-3 induction as a function of the course of successive divisions

Table I shows the SCE frequency induced in three subsequent divisions by exposure to ENU at different times with respect to administration of the first BrdU dose. The data indicate that ENU induces a significant increase in SCE-1 with respect to the matching control from 3 to 12 h. At 18, 24 and 30 h, the frequency decreases to values as low as the control values. With regard to SCE-2, the frequencies are significantly higher than the control from 3 to 24 h, with a maximum at 18 h. The SCE-2 frequency at 30 h is similar to that of the control. The SCE-3 frequencies are significantly higher at all times, even at 3 h, although there is a greater increase at 24 and 30 h.

The increments in SCE induction shown in Table I are plotted in Figure 2; two scales are used because the induction of SCE-1 is substantially lower than those of SCE-2 and SCE-3. It is important to point out the following: 9 h was the time of maximum SCE-1 frequency but had the minimum values of SCE-2 and SCE-3; 18 h was the time of maximum induction of SCE-2, but when the minimum SCE-1 and SCE-3 levels appeared; 30 h was the time of highest SCE-3 induction, but when negligible induction of SCE-1 and SCE-2 occurred. The highest SCE induction in each cell division seems to correspond to the moment prior to DNA duplication, because after this time, the course of DNA duplication reduces the SCE frequency in the current cell division. This is because there is a greater probability that lesions are produced behind the duplication fork, i.e. in the recently synthesized strands, causing a greater number of SCEs in the subsequent division. Figure 3 shows the data plotted as a percentage of the maximum induction, considering only SCE induced in the cell division of ENU exposure. A model scheme of SCE induction in subsequent divisions could be constructed from the curves. The shapes of the curves, particularly that corresponding to SCE-2, suggest that cell division in bone marrow is continuous and that the durations of the G_1, G_2 and M phases of cell division are negligible. Under these circumstances, the data indicate that SCE occurs during S phase and specifically at the replication fork. The inferences obtained from the data that support the model imply that cell division of the cells under study lasts 9 h, instead of the 12 h inferred from the average generation time (Ivett and Tice, 1992). This means the cell cycle in the present study is out of phase with respect to its theoretical course. The synchrony is due to selection of the cell subset under analysis by BrdU administration, arrest of the cells in metaphase with colchicine at specific times and the TWDS protocol for chromatids. The results will allow us to improve the TWDS protocol *in vivo*, by determining the most appropriate times for BrdU and colchicine treatments, thus obtaining a much higher percentage of cells with TWDS.

Persistent and tenacious SCE-inducing lesions

The data from Figure 2 interpreted in terms of the model of SCE induction in successive divisions indicate the following: (i) the frequencies of SCE-1, SCE-2 and SCE-3 are preferentially increased by exposure during their corresponding cell division, implying that they are caused by recently induced DNA lesions of immediate expression and, as was previously mentioned, are a result of the progress of DNA duplication; (ii) ENU did not increase the frequency of SCE-1 on exposure during the second division (18 h), indicating that this mutagen is unable to induce 'SCE-1 like' SCE arising from SCE produced at the

Table I. Frequency of SCE (mean ± SD) in three subsequent divisions induced by exposure to ENU at different times with respect to first BrdU administration

Division	Time							C1	C2	C3	C4
	3 h (x-C3)	6 h (x-C2)	9 h (x-C1)	12 h (x-C1)	18 h (x-C2)	24 h (x-C3)	30 h (x-C4)				
First	2.9 ± 0.2 (2)	2.8 ± 0.7 (2)	2.7 ± 1.3 (2.3)	2.5 ± 0.6 (2.1)	**0.86 ± 0.4** (0.11)	**0.88 ± 0.1** (–0.01)	**0.69 ± 0.2** (0.16)	0.43 ± 0.1	0.75 ± 0.1	0.89 ± 0.2	0.53 ± 0.1
Second	6.3 ± 1.0 (2.7)	7.1 ± 0.9 (2.9)	6.0 ± 0.7 (2.2)	8.7 ± 2.5 (4.9)	12.9 ± 2.3 (8.7)	5.2 ± 0.6 (1.6)	6.7 ± 0.7 (–0.6)	3.8 ± 0.3	4.2 ± 1.1	3.6 ± 0.7	7.3 ± 1.6
Third	7.4 ± 0.6 (3.6)	7.3 ± 1.0 (2.9)	6.3 ± 0.8 (2.5)	6.5 ± 1.3 (2.7)	8.3 ± 1.1 (3.9)	10.3 ± 1.4 (6.5)	15.0 ± 1.2 (10.6)	3.8 ± 0.5	4.4 ± 0.7	3.8 ± 0.6	4.4 ± 0.8
n	5	5	3	4	5	5	4	3	4	5	3

All values were statistically significant with respect to their current controls by Student's *t*-test, $P < 0.05$, except for the values in bold. C1–C4 represent the different matching control groups. x-C(1–4) represent the increase with respect to its respective matching control.

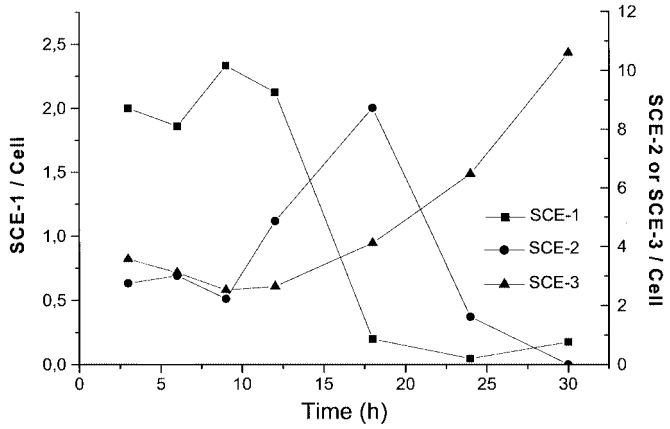

Fig. 2. Induction of SCE-1, SCE-2 and SCE-3 by exposure at different times during three cell divisions after the first BrdU administration (time 0). The data represent the increase in SCE with respect to the current control, derived from the results shown in Table I. Two scales are used because SCE-1 induction is substantially lower than SCE-2 and SCE-3.

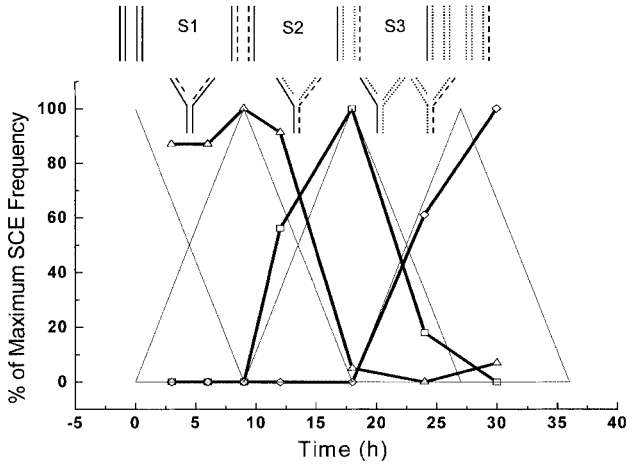

Fig. 3. Model of SCE induction in subsequent divisions. Thick lines represent the actual SCE induction in three subsequent divisions, plotted as a percentage of the maximal induction at each cell division with respect to time; the fine lines represent the theoretical progress of SCE induction in the three cell divisions. The corresponding course of subsequent DNA duplications and BrdU incorporation are shown. Continuous lines represent unsubstitution, dashed lines low BrdU substitution and dotted lines high BrdU substitution.

same locus in the second and third divisions by a tenacious DNA lesion (see Figure 1); (iii) ENU is capable of inducing a similar increase in SCE-2 and SCE-3, even higher than that of SCE-1, by exposure at different times during the first cell cycle. This result could not be explained by the presence of very persistent DNA lesions that are by chance expressed as SCE in successive cell divisions.

In order to explore the observation that ENU can produce SCE in three subsequent divisions after exposure, animals were s.c. injected with the same previously used dose of ENU, at 3, 9 or 12 h before the first BrdU administration, which means up to nearly five cell divisions prior to the analysis. The results in Table II show that for all times the frequencies of SCE-1, SCE-2 and SCE-3 were significantly different from those of their corresponding controls; they also reveal that the increase in SCE-1 frequency was lower than that for SCE-2 and that the latter was lower than the increase in SCE-3. Exposure to ENU 3 and 6 h before the first BrdU administration increases SCE in the three subsequent cell divisions by very similar proportions (1.4:2:3), while exposure 12 h before BrdU administration increases SCE-1 frequency by 1.0 and SCE-2 and SCE-3 frequencies by ~3.0. In fact, the increase in SCE-3 is practically constant. This confirms that ENU is capable of inducing SCEs for several cell divisions after exposure in cells with unsubstituted DNA and that this induction increases in the subsequent divisions after ENU exposure.

Effect of BrdU incorporation into DNA on the induction and persistence of SCE-eliciting lesions

The maximum induction of SCE-2 and SCE-3 is nearly four and five times higher than the maximum SCE-1 induction, as shown in Table I and Figure 2. This seems to indicate that BrdU incorporation is playing an important role in the sensitivity of cells to SCE induction by ENU, particularly of immediate expression lesions. Theoretically, in the course of DNA duplication (Figure 3) at 9 h DNA would be unifilarly BrdU substituted with low BrdU. After this time and up to 18 h, two kinds of DNA double strands should be formed: both would have a single strand substituted with high BrdU, but one with a complementary unsubstituted single strand and the other with a complementary low BrdU substituted strand. At 30 h, three kinds of double strands should be formed: two similar to those of the second division, but another with a high BrdU bifilar substitution. Under such circumstances, the induction of SCE seems to depend on BrdU incorporation. In order to explain how BrdU incorporation affects SCE induction by ENU, the relationship between BrdU incorporation and SCE frequency per chromosome during the three cell divisions is shown in Table III. The incorporation of BrdU was indirectly estimated as the number of substituted strands multiplied by the BrdU dose administered during substitution of these strands. The data at the times of maximum induction were used assuming that at those times duplication for each cell division had been completed. The data indicate that SCE induction is proportional to BrdU incorporation. Figure 4 shows that the relationship is directly proportional to dose, with $r = 0.99$. The estimated SCE induction by 25 mg/kg ENU in the absence of BrdU was 1.9 SCE/cell.

Table IV and Figure 5 show the frequencies of SCE-3D and SCE-3P, i.e. of chromosomes generated from the unsubstituted and BrdU-substituted strands present after the first division, respectively. Data from exposure to ENU either before or after the first BrdU administration were considered. The curves for SCE-3P seem to show a dependence on BrdU incorporation. In the first and second divisions there is a small but significant increase in SCE with respect to the control, except for the value obtained at 6 h. The fact that this BrdU-substituted strand does not exist at these times of exposure implies that some ENU-induced lesions in the unsubstituted DNA could make the newly synthesized BrdU-substituted DNA strands prone to SCE induction in subsequent divisions. Exposure during the second and third divisions continuously increases the frequency of SCE-3P.

The induction of SCE-3D was statistically significant with respect to the controls at all times of treatment. The curve for SCE-3D induction was much more complex. In the cell cycle prior to incorporation of BrdU there was an increase proportional to time. These long-lasting lesions induced during the first and second cycles are mainly SCE-3D; although this was expected, the event confirms that ENU can in fact induce long-lasting lesions in native DNA. After the first increase,

Table II. Frequency of SCE (mean ± SD) in three subsequent divisions induced by exposure to ENU at different times prior to first BrdU administration

Division	Time			C1	C2
	–3 h (x-C1)	–6 h (x-C1)	–12 h (x-C2)		
First	2.0 ± 0.5 (1.35)	2.1 ± 0.3 (1.45)	1.5 ± 0.28 (0.99)	0.65 ± 0.11	0.51 ± 0.11
Second	6.9 ± 0.9 (1.8)	7.1 ± 0.7 (2.0)	7.4 ± 0.83 (3.2)	5.1 ± 0.26	4.2 ± 0.43
Third	7.4 ± 0.7 (3.2)	7.3 ± 0.8 (3.1)	6.4 ± 0.88 (2.8)	4.2 ± 0.68	3.6 ± 0.5
n	6	6	4	6	4

All values were statistically significant with respect to their current controls by Student's *t*-test, $P < 0.05$. C1 and C2 represent the different matching control groups. x-C(1 or 2) represent the increase with respect to its respective matching control.

Table III. Effect of subsequent BrdU incorporation into DNA on SCE induction by ENU

Division	BrdU incorporation	SCE/cell
First	0.4	2.3
Second	4.2	8.7
Third	6.1	10.6

The 30 h exposure was used for the SCE-3 comparison.

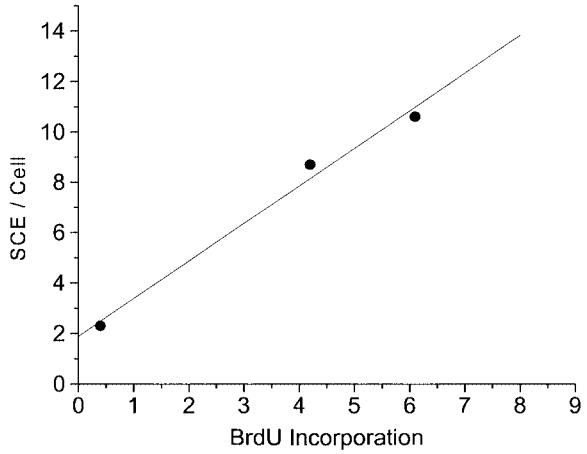

Fig. 4. Dose–response curve of BrdU incorporation versus SCE/cell. The plotted data correspond to the higher SCE induction in each cell division. The incorporation was indirectly estimated considering the number of substituted strands (Figure 3) multiplied by the BrdU dose, i.e. low 0.2 and high 2.0.

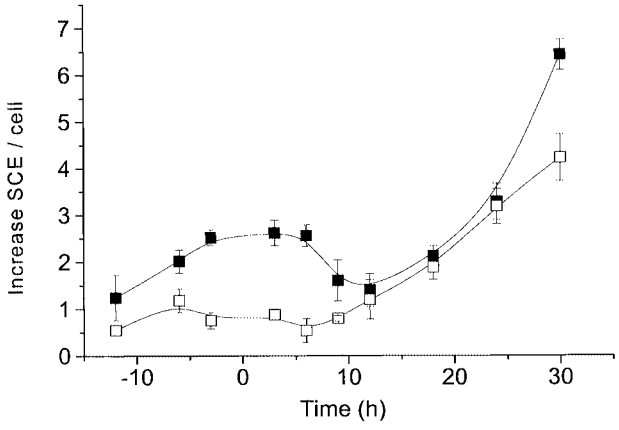

Fig. 5. Induction of SCE-3D (■) and SCE-3P (□) derived from the unsubstituted and the BdU-substituted strands, respectively, at different times before and after first BrdU administration (time 0).

the frequency remains constant, then falls at the time at which the first DNA duplication occurs, according to the previously mentioned model of cell division; this could be explained by a lower probability of SCE induction, resulting from a 50% reduction in the number of unsubstituted strands per cell. SCE-3D frequency rises again at the time at which the second DNA duplication occurs, in the presence of the high BrdU dose. Then the frequency of SCE-3D increases at a similar rate to SCE-3P; this suggests that mid-lasting lesions are not dependent on BrdU incorporation, perhaps because the difference in substitution between the two kinds of strands is minor. However, at 30 h the frequency of SCE-3D is substantially higher than that of SCE-3P. This latter behavior is not easily explained; the fact that SCE-3P and SCE-3D were scored in the same cell implies that all experimental conditions were the same and that the only difference between the dark and the pale chromosomes is the unsubstituted DNA strand of the dark chromosome and the slightly substituted strand in the pale chromosome.

Discussion

SCE-1, SCE-2 and SCE-3 induction as a function of the course of successive cell divisions

There is evidence relating SCE production to the process of DNA synthesis (Kato, 1974; Wolff *et al*., 1974) and to the inhibition of enzymes involved in this process (Ishii and Bender, 1980; Nishi *et al*., 1982), even suggesting that the replication fork is the site of SCE production (Kato, 1980; Cortés *et al*., 1993).

The results presented here indicate that the bone marrow cell subset under study could be considered synchronized, because the maximum SCE induction in each cell division corresponds to the minimum SCE frequencies of the other divisions and the maximum increases seem to occur at nearly regular periods. This synchrony is due to the fact that we selected metaphases showing TWDS derived from cells which incorporated two different BrdU doses at specific times during three cell divisions and were in metaphase at the moment of colchicine administration. The estimated duration of the bone marrow cell cycle of ~9 h agrees with a previous study in rat (Schneider *et al*., 1977).

The fact that SCE induction by ENU decreases as DNA duplication progresses, while the yield of SCE in the subsequent division rises, is direct evidence that the replication fork is the site of SCE production. This implies that the production of SCE-inducing lesions increases behind the duplication fork as DNA division elapses, reducing SCE occurrence in the current division and increasing SCE induction in the next.

Table IV. Increase in SCE frequency (mean ± SD) in third division chromosomes derived from unsubstituted (3D) and BrdU-substituted (3P) DNA strands

	–12 h	–6 h	–3 h	3 h	6 h	9 h	12 h	18 h	24 h	30 h
3D										
Treated	4.0 ± 0.8	3.7 ± 0.6	4.2 ± 0.4	4.9 ± 0.6	4.6 ± 0.6	3.8 ± 0.8	3.6 ± 0.7	4.3 ± 0.5	5.6 ± 0.9	8.5 ± 0.7
Control	1.8 ± 0.3	1.7 ± 0.6	1.7 ± 0.3	2.3 ± 0.5	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.3 ± 0.5	2.1 ± 0.4
Increase	2.2	2.0	2.5	2.6	2.4	**1.6**	**1.4**	2.1	**3.3**	6.4
3P										
Treated	2.4 ± 0.2	3.7 ± 0.6	3.3 ± 0.4	2.4 ± 0.2	*2.6 ± 0.7*	2.5 ± 0.1	2.9 ± 0.8	4.1 ± 0.5	4.7 ± 0.8	6.5 ± 1.0
Control	1.9 ± 0.3	2.5 ± 0.6	2.5 ± 0.6	1.5 ± 0.2	2.1 ± 0.4	1.7 ± 0.2	1.7 ± 0.2	2.3 ± 0.4	1.5 ± 0.2	2.3 ± 0.5
Increase	0.5	1.2	0.8	0.9	0.5	**0.8**	**1.2**	1.8	**3.2**	4.2
n	4	6	6	5	5	3	4	5	5	4

All values were significantly different with respect to the current controls, with the exception of the value in italic (3P at 6 h). The differences in SCE increases between dark (3D) and pale (3P) chromosomes were significant ($P < 0.05$) by paired *t*-test, with the exception of values in bold.

The fact that SCE takes place at the site where there is a lesion refutes the models of SCE induction caused by multiple lesions (Painter, 1980; DuFrain, 1981) and supports that which assumes that SCE occurs as a result of discreet lesions (Comings, 1975; Kato, 1977; Shafer, 1977; Dillehay *et al.*, 1989; Holden *et al*., 1989; Morales-Ramírez *et al*., 1990).

Persistent and tenacious SCE-inducing lesions

The two-tone BrdU protocol of differential staining of sister chromatids requires two cell divisions and does not allow one to detect the occurrence of SCE in each of the divisions. In addition, SCEs occurring at the same locus in subsequent divisions could be cancelled, which means that they cannot be scored because the chromosome appears as not having a SCE (Stetka, 1979); therefore, this introduces additional uncertainty in the interpretation. In fact, there are several studies in which the interpretation was based on the comparison of SCE induction by exposure to mutagens in the first division with that in the second division, inferring by difference SCE induction in each cell cycle (Ockey, 1981; Conner *et al*., 1984; Kaina and Aurich, 1985). However, the SCE obtained by exposure of unsubstituted DNA in the first cell division represent SCE induction during two cycles and the possibility of cancellation is open, while exposure in the second division represents SCE produced during one cell division but in unifilarly BrdU-substituted DNA. This underlines the importance of developing protocols that allow one to distinguish SCE occurring in each of the cell divisions, such as the TWDS method (Schvartzman and Goyanes, 1980) and others (Ishi and Bender, 1978; Latt and Loveday, 1978; Linnainma and Wolff, 1982).

The TWDS protocol introduces uncertainty in interpretation when it is applied to asynchronous, continuously dividing cells, due to the fact that lesions produced behind the replication fork will not cause SCE until the next cell division. This is particularly important when a single time is used to infer the fate of the DNA lesions involved in successive divisions (Morales-Ramírez *et al*., 1990, 1992, 1995). The present study allows us to determine the occurrence of SCE in successive cell divisions and to relate SCE induction to progress of the cell cycle, since most of the lesions eliciting SCE are of immediate expression. In fact, the results presented here indicate that ENU does not induce 'SCE-1 like' SCE as a result of SCE produced in the second and third divisions at the same locus by persistent and tenacious lesions. Under such circumstances, an unexpected increase in 'SCE-1' should be produced by a treatment during the second division. The fact that ENU does not cause SCE in the same locus contradicts our earlier results obtained by exposure to other agents. Those results showed a slight but significant increase in 'SCE-1 like' SCE, although the possibility of recent lesion induction was also considered (Morales-Ramírez *et al*., 1988, 1990, 1992, 1995). However, such studies were carried out by inducing SCE after a single mutagen treatment, at 12 h after the first BrdU dose; we assumed that at this time the first cell division had ended and therefore a true SCE-1 could not be induced. Nevertheless, the present experiment indicates that at this time there exists the possibility of causing true SCE-1. In light of the present study, 18 h is the most appropriate time for mutagen exposure so that one may study the possibility that lesions are able to cause SCE in the same locus in subsequent divisions. The fact that there is no SCE induction at the same locus in the second and third divisions implies that SCE-eliciting lesions induced by ENU do not transcend SCE occurrence. Perhaps the lesions are repaired by virtue of SCE occurrence; in fact, SCE have been related to post-replication repair (Cleaver *et al*., 1999; Limoli *et al*., 2000). Another possibility is that SCE are repaired during the interphase of the subsequent division. The data support the view that lesions are not able to cause SCE in several divisions, but contrast with earlier results in hamster cells *in vitro* and bone marrow *in vivo*, in which the level of SCE remains high throughout multiple divisions after exposure (Latt and Loveday, 1978; Morales-Ramírez *et al*., 1984). This could be explained by cell subpopulations having different cell cycle durations or, in the case of experiments *in vivo*, by the emergence of defective stem cell division, which replaces the cells killed.

Another explanation for this contradiction could be that the lesions caused by psoralen plus UV and γ-rays, which were the agents used in the previously mentioned studies, display a behavior different from those caused by ENU. However, a study carried out in bone marrow cells of mice, using ENU as the mutagen, indicated that this agent is able to increase SCE frequency when it was given even four cell divisions prior to analysis. These results are more in agreement with our observations that mutagen treatment of cells with unsubstituted DNA with ENU can produce SCE even up to five cell divisions later. This implies that other agents than ENU can also generate long-lasting increases in SCE, not by persistent lesions in DNA, but rather by another mechanism (Charles *et al*., 1986).

Our data indicate that in addition to the ENU-induced lesions which elicit SCE immediately, i.e. during the same cell division as mutagen exposure, ENU is capable of a constant induction of SCE-2 and SCE-3 by exposure in the first division or even two cell cycles prior to the first division. This could

be interpreted as ENU induction of long-lived lesions in unsubstituted DNA. There is earlier evidence of persistent DNA lesions induced by ENU that are related to SCE production. Proof of such an event was obtained in unsubstituted DNA during G_1 in salivary gland cells (González-Beltrán and Morales-Ramírez, 1999) and for even longer periods in murine peripheral blood and spleen lymphocytes (Tucker *et al*., 1986), as well as in bone marrow cells (Charles *et al*., 1986). The aforementioned data obtained in bone marrow cells imply that besides being persistent, ENU-induced lesions are capable of transcending cell division. However, the data presented here demonstrate the induction of a nearly constant frequency of SCE in each of the three successive divisions. The fact that the damage induced in the unsusbtituted DNA strands was able to cause SCE even four or five cell cycles later is relevant to the biological meaning of the SCE phenomenon. The topic of lesions causing SCE several divisions later is interesting because, besides its association with cancer (Marginson and Kleihues, 1975), it has basic implications worth mentioning. The most important ones are that the original lesion must be compatible with DNA duplication and that a secondary lesion must be produced (Kaina and Aurich, 1985). A secondary lesion capable of inducing SCE in a subsequent division could be an attractive alternative, but there remains the problem of how to explain the persistence or even the increase in SCE frequency over several divisions.

With regard to this point, it was shown that agents that inhibit DNA methylation are the only ones so far reported that cause an inheritable increment in SCE which remains constant in subsequent divisions (Perticone *et al*., 1997; Albanesi *et al*., 1999). Although the mechanism is not known, it has been suggested that SCEs are associated with DNA demethylation by increasing misligation, due to a decreased ability of DNA polymerase to discriminate between the parental and newly synthesized strands. In the case of ENU, the constant induction of SCE in subsequent divisions could be produced by the indirect demethylation of DNA caused by the removal of methylated cytosines during repair. If this process of SCE induction can be generalized to other alkylating agents, the fact is relevant because DNA methylation has been related to several events, such as regulation of gene expression, cell differentiation, aging, etc. (Holliday, 1985; Jones and Vogt, 2000). Because the analysis of SCE induction by demethylating agents was carried out using the usual two-tone protocol, it would be important to determine actual SCE induction during each cell cycle and the manner in which subsequent BrdU substitution affects this induction using the protocol employed in the present study.

The conclusions regarding the relationship between SCE induction and repair of DNA lesions involved in this process in subsequent divisions are as follows: (i) SCE induction by ENU is mainly expressed in the division in which the exposure occurs, indicating that lesions are repaired before the subsequent division; (ii) ENU does not cause lesions capable of inducing SCE at the same site in subsequent divisions in BrdU-substituted DNA, indicating that they are repaired as a consequence of SCE or during the subsequent cell division; (iii) ENU gives rise to long-lasting induction of SCE in unsubstituted DNA, producing a nearly constant increase even five cell divisions after exposure, an event which could not be explained by DNA lesions, but by DNA demethylation, i.e. caused during repair.

Effect of BrdU incorporation into DNA on the induction and persistence of SCE-eliciting lesions

The use of BrdU simplified and permitted accurate scoring of SCE, but it has introduced uncertainty regarding the effect of incorporating this analog on SCE induction by mutagens. The results of the present study, as of our earlier investigations using the TWDS protocol, clearly indicate that BrdU incorporation into DNA increases the sensitivity of cells to SCE induction by exposure to mutagens (Morales-Ramírez *et al*., 1990, 1992, 1995) and that there is a direct relationship between the number and degree of BrdU substitutions in the strands and the frequency of SCE. BrdU incorporation plays an important role in the sensitivity of cells to SCE induction by ENU, particularly in lesions of immediate expression. Mitomycin C and BrdU substitution have shown an additive effect on SCE induction, yet evidence has been reported that BrdU also sensitizes DNA to the effects of mutagens (Schvartzman and Tice, 1982; Morgan and Wolff, 1984; González-Beltrán and Morales-Ramírez, 1999). In contrast, an inhibitory effect of BrdU substitution was reported for MNNG (Popescu *et al*., 1980) and for MMS (Ockey, 1981). With respect to ENU in particular, a recent report indicated that BrdU incorporation sensitizes DNA to the induction of SCE by ENU in salivary gland cells, however, these lesions were efficiently repaired during G_1 (González-Beltrán and Morales-Ramírez, 1999). The mechanism of BrdU sensitization has not been established, but it is probably related to reaction of ENU with the additional nucleophilic site represented by the bromine atom.

Although there seems to be a direct relationship between SCE induction by ENU and BrdU incorporation, it was possible to infer that ENU is able to induce SCE in the BrdU-unsubstituted strand. This refutes the idea that BrdU is responsible for all SCE induction by the agents.

The conclusions with respect to the effect of BrdU incorporation on the expression of lesions as SCE are as follows: (i) ENU induces SCE in BrdU-unsubstituted DNA; (ii) BrdU incorporation into DNA increases the sensitivity to SCE induction by ENU and this seems to be directly proportional to BrdU substitution; (iii) ENU increased SCE in BrdU-substituted chromosomes slightly but significantly, even when the treatment occurred before BrdU incorporation.

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