Segregation of DNA polynucleotide strands into sister chromatids and the use of endoreduplicated cells to track sister chromatid exchanges induced by crosslinks, alkylations, or x-ray damage
(chromosomes/repair/DNA damage)

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ABSTRACT The method of Matsumoto and Ohta [Matsumoto, K. & Ohta, T. (1992) Chromosoma 102, 60–65; Matsumoto, K. & Ohta, T. (1995) Mutat. Res. 326, 93–98] to induce large numbers of endoreduplicated Chinese hamster ovary cells has now been coupled with the fluorescence-plus-Giemsas method of Perry and Wolff [Perry, P. & Wolff, S. (1974) Nature (London) 251, 156–158] to produce harlequin endoreduplicated chromosomes that after the third round of DNA replication are composed of a chromosome with a light chromatid and a dark chromatid in close apposition to its sister chromatid containing two light chromatids. Unless the pattern is disrupted by sister chromatid exchange (SCE), the dark chromatid is always in the center, so that the order of the chromatids is light–dark–light–light. The advent of this method, which permits the observation of SCEs in endoreduplicated cells, makes it possible to determine with great ease in which cell cycle an SCE occurred. This now allows us to approach several vexing questions about the induction of SCEs (genetic damage and its repair) after exposure to various types of mutagenic carcinogens. The present experiments have allowed us to observe how many cell cycles various types of lesions that are induced in DNA by a crosslinking agent, an alkylating agent, or ionizing radiation, and that are responsible for the induction of SCEs, persist before being repaired and thus lose their ability to inflict genetic damage. Other experiments with various types of mutagenic carcinogens and various types of cell lines that have defects in DNA repair processes, such as mismatch repair, excision repair, crosslink repair, and DNA-strand-break repair, can now be carried out to determine the role of these types of repair in removing specific types of lesions.

When cells are exposed to ultraviolet radiation (1) or to chemical mutagenic carcinogens (2), one of the most readily observable effects is the induction of chromosomal exchanges between sister chromatids. If the DNA is allowed to replicate in the presence of the thymidine analog 5-bromodeoxyuridine (BrdUrd), in which the methyl group of the thymidine is replaced with the heavier atom bromine, then, because of the semiconservative replication of DNA, each sister chromatid contains one original light polynucleotide strand and one new heavy strand containing BrdUrd. If such a chromatid replicates again in the presence of BrdUrd, each polynucleotide strand separates from its complementary strand and is now paired with a new heavy strand. This results in chromosomes in which one sister chromatid is bifilarly labeled with BrdUrd, and the other sister chromatid is only unifilarly labeled. The two sister chromatids are now chemically different from one another and can be made to stain differentially (3). The chromatid containing more BrdUrd always stains lighter than its sister. Any sister chromatid exchange (SCE) can now be seen clearly with great resolution (4). In fact, the induction of SCEs visible in such chromosomes has constituted one of the most sensitive mammalian tests for the effects of mutagenic carcinogens (5).

Because cells must replicate at least twice before an SCE becomes visible, any given SCE could have been induced at either of the S periods before the cells reach the metaphase at which the exchanges are scored (1). In cells exposed to chemical agents, this has raised questions of how long any lesion, or adduct, remains unrepaired in the DNA and therefore remains capable of producing an SCE.

In previous attempts to determine in which cell cycle an SCE was actually formed, cells often were cultured for three rounds of replication in the presence of BrdUrd, which led to three-way differential staining of the chromosomes (6–9). This method frequently is subjective because of the vagaries in stain intensity brought about by BrdUrd depletion, which leads to varying amounts of BrdUrd being incorporated in each round of replication, as well as by other complications of the fluorescence-plus-Giemsas (FPG) staining technique.

In Chinese hamster ovary (CHO) cells that have already undergone two rounds of DNA replication, we have now induced chromosomes to undergo endoreduplication at high frequency (~65%) by treating the cells with rotenone according to the method of Matsumoto and Ohta (9, 10). If the replication cycle that results in endoreduplication takes place in the absence of BrdUrd, then the endoreduplicated chromosome pairs consist of one chromosome in which both chromatids are unifilarly substituted and will stain lightly by the FPG technique, and one chromosome that contains a darkly stained sister chromatin with unsubstituted DNA and a lightly stained chromatin with unifilarly substituted DNA (Fig. 1). A similar staining pattern is obtained even if BrdUrd is present in the replication cycle that results in endoreduplication, but in this case the light chromatid will be bifilarly substituted and the dark chromatid will be unifilarly substituted. Because of the topological constraints that lead to the newly replicated polynucleotide strand always segregating to the outside of a pair of sister chromatids (4, 11–13), in either case the endoreduplicated pair then consists of a chromosome with a light chromatid and its light sister chromatid lying next to a dark chromatid from the chromosome containing a dark chromatid and a light sister chromatid (Fig. 2). Thus the order of the chromatids in such an endoreduplicated pair of chromosomes is light–dark next to light–light, with the dark chromatid always in the center. An SCE will disrupt this pattern. Furthermore, in such cells, by using the criteria of Brewen and Peacock (14) that were established for SCEs

Abbreviations: SCE, sister chromatid exchange; FPG, fluorescenc-plus-Giemsas; CHO, Chinese hamster ovary; MMC, mitomycin C; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; BrdUrd, 5-bromodeoxygenidine.

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visualized in autoradiographs, it is now extremely easy to
determine at which round of DNA replication the SCE actually
occurred. Any SCE that occurs in the first cycle of replication
interrupts the pattern described above so that the exchange
coccurs between the two inner chromatids of the endoredupli-
cated pair—i.e., between the darkly stained inner chromatid
and the lightly stained inner chromatid of the other chromo-
some (Fig. 3). This is a between-chromosome, inner-inner
exchange (14). If the SCE occurs in the second cycle, then it
is also manifested as an exchange between chromatides, but
now it is between the darkly stained inner chromatid and the
outer chromatid of the lightly stained pair—i.e., is a between-
chromosome, inner-outer exchange (Fig. 3). When an SCE
occurs in the third round of replication, which leads to the
endoreduplication, it is discernible only within the chromo-
some that contains one dark and one light chromatid and does
not occur between the paired chromatides. It is thus classified
as a within-chromosome SCE, rather than between-
chromosome SCE (Fig. 3). It is thus possible to tell with great
precision and ease at which division the SCE occurred (Fig. 2).

A similar technique has been developed independently by R.
Meschini, R. Bastianelli, and F. Palitti (personal communica-
tion), who use a different terminology to describe first-, second-, and third-division SCEs.

On rare occasions we have seen an endoreduplicated chromo-
some pair that has the dark chromatid on the outside. This
discrepant arrangement is evidently an artifact that arises when
the metaphase plate is flattened during drying of the slides. In
one of the cells in Fig. 2, a pair of crossed chromosomes can
be seen. If the chromosome with the dark chromatid slides in
one direction to lie parallel with its sister chromosome, then
the light–dark light–light orientation will be maintained. If,
however, it slides the other way, then the dark chromatid will
be on the outside. In addition, a third-division SCE will move
portions of a dark chromatid to the outside. To avoid confu-
sion, it is therefore necessary to score third-division (i.e.,
within-chromosome) SCEs first to visualize the position of the
dark chromatid in the previous cycle.

Experiments with various chemical mutagenic carcinogens
and x-rays have now been carried out in which treatments that
induce SCEs are administered only in the first cell cycle. From the pattern of SCEs visible in the endoreduplicated chromosomes, it is possible to obtain answers to several persistent questions that have arisen about the induction of SCEs. For instance, it is possible to determine how long (for how many cell cycles) the lesions persisted and thus inflicted genetic damage. By using cell lines with different defects in DNA repair, it will also be possible to determine how various defects in DNA repair contribute to the damage manifested as SCEs.

**MATERIALS AND METHODS**

CHO cells were cultured in Ham's F12 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units of penicillin per ml, and 100 \( \mu \)g of streptomycin per ml at 37°C in a 5% CO\(_2\) incubator.

Endoreduplication was induced in these cells by a slight modification of the method of Matsumoto and Ohta (9, 10), described as follows. For each treatment, four 100-mm Petri dishes were seeded with 10\(^6\) cells each. After 24 hr, the cultures were exposed to various doses of x-rays (100 cGy/min) from a Philips RT250 therapeutic x-ray unit (250 kVp, 15 mA, half-value layer 1.06 mm Cu) or were exposed to various doses of mitomycin C (MMC) or \( N \)-methyl-\( N' \)-nitro-\( N \)-nitrosoguanidine (MNNG) for 1 hr. After two washes in phosphate-buffered saline (PBS), the treatments were followed by the addition of fresh medium containing BrdUrd (final concentration, 20 \( \mu \)M). When the cells had undergone two cell divisions in the presence of BrdUrd (=25 hr), Colcemid (Sigma) (final concentration, 2 \( \times \) 10\(^{-7}\) M) was added to the cultures for 1 hr. The cells were then washed twice with PBS and incubated in fresh medium for 20 min to allow the cells time to form the mitotic spindle. At this time, 5 \( \mu \)g/ml of rotenone (Aldrich; 2 mg/ml stock solution in dimethyl sulfoxide) was added to the dishes and the mitotic cells were immediately dislodged by gentle pipetting. The suspension from each of the four plates was pooled and incubated for 3 hr at 37°C. This was followed by washing the cells twice with PBS, resuspending them in fresh medium, and replating cells in a 100-mm Petri dish for 24 hr. Two hours before fixation, Colcemid (final concentration, 2 \( \times \) 10\(^{-7}\) ) was added to the cultures to accumulate cells in metaphase.

The mitotic cells, including the endoreduplicated cells, were collected by shaking off the metaphase cells (15) and were then centrifuged. The pellet was resuspended in 0.075 M KCl for 5 min at 37°C. After recentrifugation, methanol was added to the pellet, followed by two washes in fixative (methanol-acetic acid, 3:1). Cells were dropped onto wet slides and air dried. Virtually all cells on the slide were metaphase plates and \( \approx 65\% \) of these were endoreduplicated.

The slides were stained by a modified FPG technique (3). Briefly, the slides were immersed in Hoechst 33258 (5 \( \mu \)g/ml, final concentration) in M/15 Sørensen's phosphate buffer (pH 6.8) for 20 min. The slides were then washed in distilled water, and coverslips were mounted with a few drops of Sørensen's buffer. Next, the slides were exposed for 12 min on a 55°C slide.

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**FIG. 3** Scheme of the staining patterns that arise after SCEs are produced in the first, second, or third cycle of DNA replication. SCEs formed in the first cycle are between the inner chromatids of the paired chromosomes (between, inner–inner); those formed in the second cycle are between the inner dark chromatid of one chromosome and the outer chromatid of its sister (between, inner–outer); those formed at the third cycle are within a single chromosome (within).
warming tray to black light from two GE BLB tubes, and stained in 5% Giemsa in Sørensen’s buffer for 5–8 min.

**RESULTS AND DISCUSSION**

It has been proposed that when DNA is crosslinked, SCEs are produced in accord with a crosslink bypass model rather than by usually accepted models of repair (16). This model postulates that when a DNA replication fork meets a crosslink in DNA, replication bypasses the crosslink by replicating across the link to the now connected complementary polynucleotide strand. Thus, the crosslink is not removed from one of the daughter chromatids, but the sister chromatid, after breakage and reunion of its polynucleotide strands, does have a switch of label that will subsequently lead to an SCE. According to this model, because of the way the label is distributed between the crosslinked polynucleotide strands at the second round of replication, a visible SCE would not be produced in one of the two daughter chromosomes found in cells made tetraploid after the first replication—i.e., no twin SCEs (17) would be found (18). Previous experiments (19) with the crosslinking agent MMC, however, have shown that at the concentrations used in the present experiments, MMC does indeed induce twin SCEs observed in the tetraploid cells, and that the predictions of the bypass model do not occur. Experiments have now been carried out with MMC to see if repair of crosslinks that induce SCEs actually occurs as the cells divide. The experiments with endoreduplicated cells show that after a 1-hr exposure to 50 nM MMC, SCEs were increased only in the first cycle after treatment (Table 1). That is, the inner-inner SCEs increased from a background level of 155 in 40 cells to 179 in 20 cells (P << 0.001, Student’s t test). The numbers of SCEs found in the second and third divisions did not differ significantly (P = 0.22–0.34) from those in the controls. These results with entire chromosome complements of the cells corroborate the findings of Linnainmaa and Wolff (19), which were obtained by using only a single unpaired chromosome from each cell. The results indicate that normal CHO cells are able to repair DNA crosslinks, and that SCEs are no longer induced after repair of the lesions.

SCEs, however, can be induced very efficiently by other mutagenic carcinogens, including simple alkylating agents such as MNNG and can also be induced, albeit less efficiently, by agents that mainly induce strand breaks in DNA, such as x-rays. Therefore, similar experiments were carried out with MNNG and with x-rays to see if the cells handled, or repaired, their SCE-inducing lesions differently from the way they handled crosslinks.

When the cells were exposed before the first S period to very low concentrations of MNNG for only 1 hr, a large number of SCEs were induced in the first cycle (Table 1). A similar number were induced in the second cycle as well, and SCEs were still being induced even in the third cycle. Clearly, the lesions induced by the alkylating agent MNNG are not being repaired as efficiently as are crosslinks.

When the cells were exposed to x-rays, which mainly induce DNA strand breaks, very few SCEs were induced until high doses that induce discernible base damage had been administered (Table 1). At 250 cGy an increase in SCEs was produced only in the first cycle. After 500 cGy of x-rays, however, the first-cycle SCEs were decreased 2-fold. In the second cycle, a significant increase in SCEs still occurred, but it was now only a 36% increase, and by the third cycle only a control level was found.

Sasaki (20) found in human embryonic fibroblasts that when endoreduplication was induced by 2-mercaptoethanol, the numbers of SCEs increased. Such was not the case, however, in Chinese hamster V79 cells in which endoreduplication was induced by hydrazine (21) or Colcemid (22). Therefore, to relate the numbers of SCEs induced in CHO cells exposed to rotenone, which induces endoreduplication at a very high rate, to the levels found in unexposed cells, a standard SCE test was carried out in which cells growing exponentially in T75 flasks were exposed to rotenone for 3 hr before the addition of

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### Table 1. SCEs induced by MMC (1 hr), MNNG (1 hr), or x rays in the first, second, or third cell cycle as seen in endoreduplicated cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells</th>
<th>No. SCEs</th>
<th>SCEs/cell</th>
<th>No. SCEs</th>
<th>SCEs/cell</th>
<th>No. SCEs</th>
<th>SCEs/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>3.88</td>
<td>132</td>
<td>3.3</td>
<td>181</td>
<td>4.53</td>
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<tr>
<td>50 nM</td>
<td>20</td>
<td>179</td>
<td>8.95</td>
<td>76</td>
<td>3.8</td>
<td>77</td>
<td>3.85</td>
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<td>MNNG</td>
<td></td>
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<tr>
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<td>4.45</td>
<td>72</td>
<td>3.60</td>
<td>88</td>
<td>4.40</td>
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<tr>
<td>75 nM</td>
<td>20</td>
<td>164</td>
<td>8.20</td>
<td>163</td>
<td>8.15</td>
<td>130</td>
<td>6.50</td>
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<tr>
<td>Control</td>
<td>20</td>
<td>84</td>
<td>4.20</td>
<td>63</td>
<td>3.15</td>
<td>76</td>
<td>3.80</td>
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<tr>
<td>60 nM</td>
<td>20</td>
<td>141</td>
<td>7.05</td>
<td>131</td>
<td>6.55</td>
<td>111</td>
<td>5.55</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>221</td>
<td>4.42</td>
<td>170</td>
<td>3.40</td>
<td>228</td>
<td>4.56</td>
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<tr>
<td>100 cGy</td>
<td>50</td>
<td>125</td>
<td>2.50</td>
<td>192</td>
<td>3.84</td>
<td>200</td>
<td>4.00</td>
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<tr>
<td>250 cGy</td>
<td>50</td>
<td>334</td>
<td>6.68</td>
<td>184</td>
<td>3.68</td>
<td>202</td>
<td>4.04</td>
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<tr>
<td>500 cGy</td>
<td>50</td>
<td>461</td>
<td>9.22</td>
<td>231</td>
<td>4.62</td>
<td>197</td>
<td>3.94</td>
</tr>
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</table>

### Table 2. SCEs induced in CHO Cells by rotenone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. SCEs/No. chromosomes</th>
<th>SCEs per cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>328/1005</td>
<td>6.56</td>
</tr>
<tr>
<td>Rotenone</td>
<td>430/1004</td>
<td>8.60</td>
</tr>
</tbody>
</table>

Treatment with 5 μg/ml rotenone for 3 hr. Fifty cells per point, P < 0.001 (Student’s t test).
BrdUrd for two cell cycles. Under such conditions no endoreduplication occurs. The experiment (Table 2) showed that the frequency of SCEs (8.60 per cell) found after two rounds of DNA replication was about 30% higher than that in untreated controls (6.56 per cell). Because treatment with rotenone increases cell cycle times, second-cycle cells appeared at metaphase 28 hr after the addition of BrdUrd in the control cells but only after 43 hr in rotenone-treated cells. Because the SCEs observed after two cycles in BrdUrd are those induced in both the first and second cycles, it appears that rotenone can induce a 15% increase in SCEs per cell cycle when cells are kept for two rounds of DNA replication after exposure. In the endoreduplication experiments, however, cells that had already undergone two rounds of replication in the presence of BrdUrd were kept for only one cycle after exposure to rotenone; therefore, any rotenone-induced increase in SCEs should have occurred only in the third cycle—i.e., the cycle that took place after treatment with rotenone. However, the data for the controls in all the endoreduplication experiments indicate that equal numbers of SCEs were found in all three cycles, and thus that rotenone did not increase SCEs at the time of endoreduplication. Nevertheless, in two of the four controls in the endoreduplication experiments, the number of SCEs was higher than that in the controls in the standard experiment. This result, which is similar to those of Sasaki (20), is most likely attributable to the control being somewhat low in the standard experiment.

The results of this study indicate that CHO cells handle the lesions induced by the three agents differently. Even at doses that induced high levels of SCEs, the crosslinking agent MMC produced lesions that lasted only one cell cycle, whereas MNNG induced lesions that were still in the DNA and capable of inducing SCEs at the third cycle. X-rays induced lesions that were intermediate; even after high doses that induced equivalent numbers of SCEs in the first cycle as do the other two agents, only a few SCEs were induced in the second cycle and none were induced in the third. The cells, therefore, removed or repaired crosslinks, simple alkylations, and the minor x-ray-induced lesions at different rates.

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