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Cycle-related toxicity and transformation in 10T1/2 cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

(synchrony/confluence arrest/colony formation/focus assay)

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ABSTRACT **Exposure of C3H 10T1/2 Cl 8 cells, synchronized by release from confluence-induced arrest of proliferation, to different concentrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 30 min at various points during the cell cycle causes dose-dependent toxicity (decrease in relative colony-forming efficiency or "survival") that increases linearly during the first G_1 phase, reaches a maximum in early to middle S phase, and decreases during late S. In the course of the second S phase, toxicity again becomes maximal. The transformation rate (type III foci) increases and decreases with a similar pattern, increasing during the first G_1 phase to a maximum during early S phase, subsequently decreasing, and then increasing again during the second S phase. Although periods of maximal toxicity and transformation roughly coincide with some portion of the S phase, the mechanisms underlying these phenomena appear to differ for the following reasons: (*a*) toxicity is linearly related to dose of MNNG, whereas the latter is linearly related to the logarithm of transformation rate, and (*b*) the ratio between toxicity and transformation varies with the cycle phase and the dose of MNNG.**

The relationship between cell proliferation and chemical carcinogenesis has been demonstrated in a number of *in vivo* (1, 2) and *in vitro* (3–7) studies. Although it is widely recognized that the proliferation of target cells directly influences the frequency of neoplastic transformation, the phase or phases of the cell cycle most susceptible to induction of transformation by various carcinogenic chemicals has not been established. Knowledge of the critical biochemical events that take place in the different phases of the cell's life cycle is increasing, and the demonstration that cells are particularly sensitive during one or more phases may help to identify the underlying biochemical mechanisms involved in the initiation and expression of neoplasia.

Because a firm understanding of intracyclic susceptibility to neoplastic transformation is important, we have examined the effect of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) applied for brief intervals at precise points in the cycle of 10T1/2 cells synchronized by release from confluence-induced arrest of proliferation. We have used procedures that allow both toxicity and transformation to be quantitated accurately and we have used doses of MNNG that produce toxicity that selectively affects 20–90% of the cell population. MNNG was chosen because it is a spontaneously activated, rapidly catabolized alkylating agent whose pattern of alkylation of DNA bases is well known (8). Synchronization by release from confluence-induced arrest of proliferation was used because it appears to cause less disturbance of cellular physiology than do other synchronization techniques (3, 9), including thymidine blockade (10, 11) and isoleucine starvation (10, 12). The results of this study indicate that the S phase of 10T1/2 cells is the period of maximal sensitivity to MNNG-induced transformation and toxicity; early S is more sensitive than late S.

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MATERIALS AND METHODS

Culture and Synchronization of Cells. C3H 10T1/2 Cl 8 cells were a gift from John B. Little (Harvard University, Boston, MA). Cells were used when they were between passages 16 and 19 *in vitro*. At the time they were used, the stock cells met all basal cytologic and growth criteria established by Reznikoff *et al.* (13). Stock cultures were maintained as described (14). Spontaneous transformation occurred at the rate of $39.4 \pm 2.0/10^6$ cells in the untreated population under the conditions of culture used in this study. Basal colony-forming efficiency (mean ± SD) was 28.5 ± 4.6% ($n = 9$). Synchronization of proliferation of cultured cells was accomplished by plating logarithmic phase cells at a concentration of 10^6 cells per 100-mm dish and maintaining them for 4 days without refeeding; at the end of this time, they had been in a fully confluent, nonproliferating state for 24 hr. Cultures were then trypsinized and the dispersed cells were counted and replated in complete medium, after which they began to proliferate synchronously (14).

Assessment of Synchronous Proliferation. To monitor the progression of 10T1/2 cells through the cell cycle after release from confluence-induced arrest of proliferation, cells were counted and the incorporation of [^{3}H]thymidine into DNA was determined. For the latter purpose, cultures were exposed to medium containing 2 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]dThd per ml for 30 min. At each time point, triplicate cultures were exhaustively washed with Hanks' balanced salt solution and treated with trypsin. Released cells were counted and lysed in scintillation fluid and the radioactivity was determined in a liquid scintillation spectrometer. The [^{3}H]dThd incorporated was expressed as cpm/10^5 cells. The fraction of cells in S phase was determined by evaluating autoradiograms prepared with liquid emulsion.

Preparation of MNNG Solutions. MNNG was obtained from Aldrich. For all experiments, a single stock MNNG solution was prepared in advance by dissolving the chemical in spectrophotometric grade acetone (Aldrich). Aliquots were placed in individual test tubes and the acetone was evaporated under a stream of N_2 gas. The tubes were sealed and kept in a desiccator at −70°C until needed. This procedure was used to minimize errors in weighing and diluting from experiment to experiment. Immediately prior to its use, a freshly thawed aliquot of MNNG was redissolved in spectrophotometric grade acetone and appropriately diluted in Hanks' balanced salt solution to the desired concentration. For exposure of cultures, the medium was removed and replaced with 5 ml of Hanks' balanced salt solution containing MNNG. All exposures were

Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; RCFE, relative colony-forming efficiency.

for 30 min, after which the balanced salt solution containing MNNG was removed, and the cultures were washed with balanced salt solution lacking MNNG and re-fed with complete medium.

Toxicity Assay. To determine toxicity, 500 cells were plated in 100-mm dishes and relative colony-forming efficiency (RCFE) was tabulated as described (14). Cultures were fed with complete medium twice weekly and maintained for 10–14 days. Triplicate cultures were treated at multiple time points after release from confluence-induced arrest of proliferation. At each time point, replicate cultures were also exposed to at least three concentrations of MNNG between 0.5 and 5 μg/ml. RCFE was also assessed at each time point in cultures exposed only to Hanks' balanced salt solution (lacking either acetone or MNNG) or to Hanks' balanced salt solution containing acetone at the concentration in MNNG-treated cultures.

Transformation Assay. The transformation frequency was determined by quantitating type III foci (15). Three concentrations of MNNG were used at each time point: low dose (reduction of RCFE by 15–25%), median dose (reduction of RCFE by 30–60%), and high dose (reduction of RCFE by 75–95%). The final concentrations of MNNG (in μg/ml Hanks' balanced salt solution) needed to obtain these levels of toxicity were determined from dose–response curves constructed from data derived from previous toxicity determinations. In all instances, the toxicity of the selected dose was reevaluated simultaneously with the transformation assay so that the exact reduction of the exposed population by each dose used for transformation studies could be quantified. Equitoxic doses were used in transformation studies for each time point during the cell cycle in each dose range.

For transformation assays, the densities at which cells were seeded were chosen to ensure that the number of viable cells per dish remaining after treatment with various concentrations of MNNG would be approximately equivalent. Plating densities used were as follows: controls (without or with acetone), 1×10^4 cells; low dose, 1.25×10^4 cells; median dose, 2×10^4 cells; high dose, 1×10^5 cells. At the time of exposure to MNNG, two plates at each dose and time were trypsinized and the cells were counted to verify cell density. In separate studies, we have found that neither the rate of toxicity nor of transformation was affected by the initial cell density when 10T1/2 cells within this range of plating densities were exposed to MNNG.

All cultures were maintained for 5 weeks after treatment; they were fed with complete medium twice weekly during this interval. On day 35, cultures were fixed with methanol/acetic acid, 3:1 (vol/vol), and stained with Giemsa stain, and the number of type III foci was determined (14, 15). For each time point, 10 replicate plates were evaluated for controls (5 with and 5 without acetone) and for each concentration of MNNG used. The number of type III foci found ranged from a low of 0–2 per 10 plates in control cultures to a high of 18–23 per 10 plates in cultures treated with median and high concentrations of MNNG at the times of maximal cell cycle sensitivity to transformation (26 and 28 hr).

Statistical Analyses. In each treatment group the frequency distribution of number of foci per plate was compared to a Poisson distribution. Although a Poisson distribution did not provide statistically satisfactory fits for the number of foci per plate, the extent of variation in these data was substantially less than would have been expected under a Poisson distribution, rather than more. In this regard, the variance for certain combined sets of distributions with similar means ranged from approximately 25% of the mean to approximately 50% of the mean, when the mean was larger than 1.0.

At all time points and doses, the SDs of rates of toxicity and transformation were less than 18% of the mean; SDs are not plotted, other than in Fig. 3, to avoid complicating the graphs.

RESULTS

Synchronous Cell Proliferation. When released from confluence-induced arrest of proliferation, the cells began to enter S phase 18–20 hr later (Fig. 1*A*). About 17 hr were needed for all cells to complete S phase. The peak rate of DNA synthesis occurred 9–10 hr after the onset of S, when autoradiographic evaluation showed that 90–96% of the cell population was in S phase. These data demonstrated that the majority of cells replicated their DNA during the S phase and that they entered the S phase at an average rate of about 10% to 12%/hr. The doubling of the cell population between 32 and 38 hr after release indicated that M phase occurred during this interval. This degree of synchronization, while not perfect, compares favorably with reports in which the extent of synchronization has been quantitated by other methods (16). Rate of entry of cells into S phase appears to provide as accurate an assessment of the degree of synchronization as do other methods.

Toxicity. The cytotoxicity of MNNG was evaluated by determining the RCFE in studies in which several concentrations of MNNG were applied to replicate cultures for 30 min at different times after release of cells from confluence-induced arrest of proliferation (Fig. 2). When exposed to MNNG shortly after release, the toxicity curve had a distinct shoulder. When cell populations were exposed to MNNG at later points in the cycle, the shoulder narrowed and the slope of the curve increased. After reaching a low point at 26–30 hr after reversal, the RCFE increased and then declined again as the second cycle reached its S phase (44 hr after release). At the concentrations used in this study, MNNG does not kill 10T1/2 cells acutely. Instead, it makes 10T1/2 cells reproductively incompetent (sterile); affected cells remain attached to the dish and they are viable as indicated by their ability to exclude trypan blue, although they are unable to proliferate.

Cell cycle effects on toxicity are clearly demonstrated in Fig. 1*B* which shows that the cycle-dependent toxicity pattern is similar at all three concentrations of MNNG. Toxicity, as indicated by a decrease in the RCFE, steadily increased during G_1 to reach a maximum during early S phase. In the course of the remainder of the first S phase, toxicity decreased (survival increased) and then increased to a second maximum in the second S phase. Basal levels of toxicity were not attained between the first and second cycles, probably because the second G_1 phase was greatly compressed. Specific toxicity (cells surviving per 10^6 cells treated per μg of MNNG) was nearly identical at low, median, and high doses. This indicated that dose and toxicity were linearly related throughout the range of concentrations of MNNG studied (Fig. 3).

Transformation. At low and median doses of MNNG, the specific transformation rates of treated cells (cells transformed/10^6 cells treated/μg MNNG) were similar (Fig. 1*C*). Unlike toxicity, the rate of transformation increased only slightly during the G_1 phase. The specific transformation rate of the treated population increased sharply with the onset of S phase and peaked at 26 and 44 hr after release for cells treated with low and median doses of MNNG. Transformation decreased markedly during the latter half of the S phase. At high doses of MNNG, the expression of transformation in the treated population was low during all phases of the cell cycle. High doses of MNNG transformed only a small fraction of the initial population because high toxicity prevented the expression of transformation in many initiated cells. The ratio of rates of toxicity and transformation in the treated population was

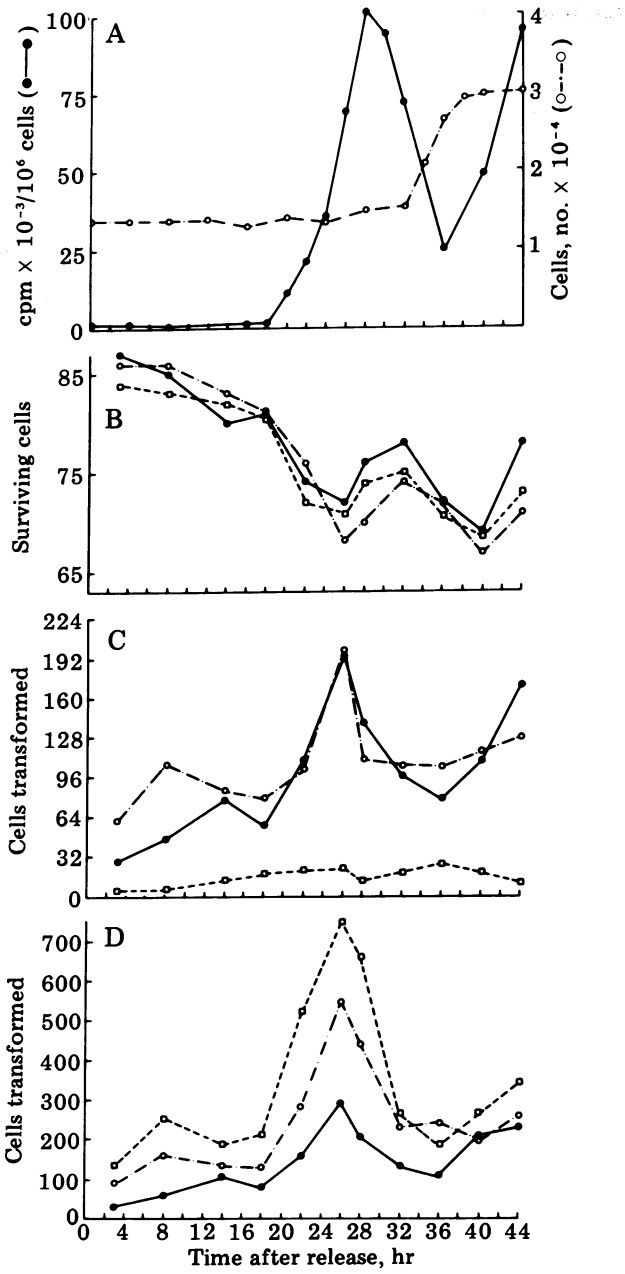

FIG. 1. Cycle-dependent events in 10T1/2 cells whose proliferation was synchronized by release from confluence-induced arrest of proliferation. (*A*) Timing of DNA synthesis and cell doubling. (*B*) Fraction of surviving cells (no. × 10^{-4}/10^6 treated) per μg of MNNG after exposure for 30 min to low, median, or high dose of MNNG at different times after release. (*C*) Transformation frequency (no./10^6 treated) of the exposed population per μg of MNNG after exposure for 30 min to low, median, or high dose of MNNG at different times after release. (*D*) Transformation frequency (no./10^6 survivors) of the surviving population of cells per μg of MNNG after exposure for 30 min to low, median, or high dose of MNNG at different times after release. For *B*, *C*, and *D*; ●—●, low dose; ○---○, median dose; □---□, high dose.

similar at both low and median doses of MNNG. Throughout the cycle 2461 ± 360 cells (mean ± SD) were sterilized and prevented from proliferating for every cell that was transformed at low doses, and this value was 2358 ± 429 at median doses, but it was 17,269 ± 1277 at high doses. These data suggest that many potentially transformed cells are prevented from expressing this property because they cannot proliferate. At high doses of MNNG, cell sterilization (inability to proliferate) vastly predominated over transformation.

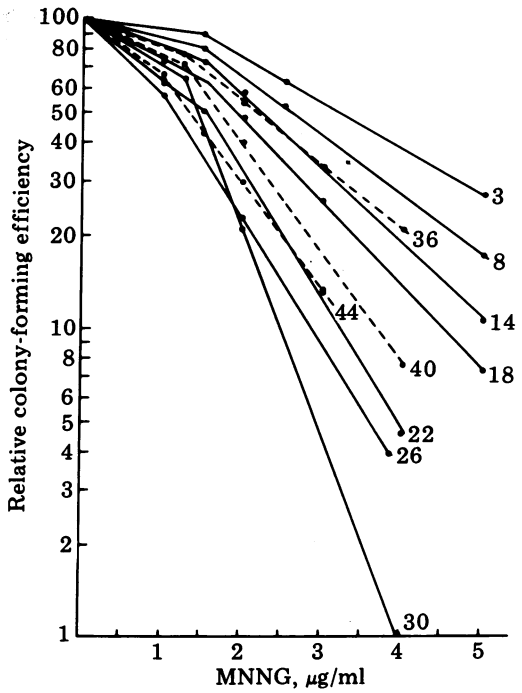

FIG. 2. Multiple-point dose–toxicity curves for 10T1/2 cells treated for 30 min with varying doses of MNNG at different times after release from confluence-induced arrest of proliferation. Numbers at the ends of the lines indicate the time (hours) after release when toxicity was tested. Solid lines indicate the first cycle and broken lines indicate the second cycle of proliferation. Toxicity is indicated by decreased RCFE.

When corrected for cell survival, these data provide an assessment of the relative risk to transformation among the population of cells surviving toxic reactions. The results (Fig. 1*D*) indicated that the risk to survivors was relatively greater at higher doses of MNNG—i.e., the relationship between dose and transformation of survivors was not arithmetically linear. This is corroborated by Fig. 3, which demonstrates that the dose of MNNG was directly related to the logarithm of the transformation rate of surviving cells. Although Fig. 3 portrays data for a single time point, 18 hr after release of confluence-arrest, the logarithm of the rate of transformation was also linearly related to dose of MNNG at other time points. The risk of transformation of surviving cells was greatest during the first

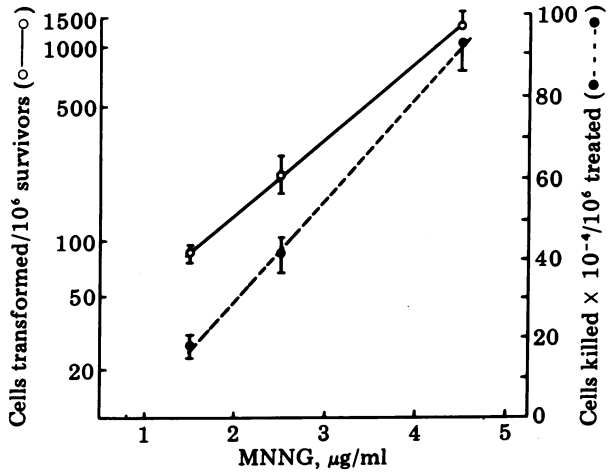

FIG. 3. Dose–response curves for cell killing and transformation at 18 hr after release of 10T1/2 cells from confluence-induced arrest of proliferation.

half of S phase and declined during the latter half of this period.

DISCUSSION

Several studies of the intracycle sensitivity of cultured cells to a few carcinogens have been described. Bertram and Heidelberger (3) reported the effect of MNNG on 10T1/2 cells synchronized by release from confluence-induced arrest of proliferation, arrest following growth in the absence of isoleucine or arginine, and arrest in the presence of high levels of thymidine. They found that the period of maximal sensitivity varied somewhat with the method of synchronization, but a period of about 4 hr preceding the G_1–S border had the greatest sensitivity in most studies. Unfortunately, interpretation of these results is hampered by Bertram and Heidelberger's decision to quantitate both toxicity and transformation in the same plate. Accurate quantitation of transformation requires that large numbers of cells per plate be exposed to carcinogen because transformation occurs at a low frequency. To yield an accurately quantifiable number of discrete colonies for toxicity assays, small numbers of cells per plate need to be exposed. In order to quantify toxicity at the high plating levels required to measure transformation, Bertram and Heidelberger used a single dose of MNNG that killed more than 99.9% of the treated cell population. Furthermore, their use of only one concentration of carcinogen did not allow the frequency of toxicity and transformation to be compared at multiple doses which may yield differing proportions of toxicity and transformation.

Marquardt (4) reported that the middle of S phase showed the most sensitivity to transformation by both MNNG and 7,12-dimethylbenz[*a*]anthracene-5,6-oxide in murine M-2 cells synchronized by release from thymidine blockade. Both early S phase (G_1–S) and late S phase were less sensitive to the induction of transformation, suggesting that susceptibility to transformation may be related to the magnitude of DNA synthesis. Exposure to multiple levels of carcinogens was used to evaluate toxicity and transformation, showing that both effects were dose-related. However, toxicity, which was maintained at below 50%, was cycle-independent, indicating that the transformation frequency was not directly influenced by the level of cytotoxicity. Furthermore, use of thymidine blockade did not allow comprehensive examination of the relative susceptibility of the G_1 phase of the cycle.

Jones *et al.* (5, 6) analyzed the cycle phase susceptibility to 5-fluoro-2′-deoxyuridine, 5-fluorouracil, and 1-β-D-arabinofuranosylcytosine of 10T1/2 cells synchronized by release from arrest of proliferation induced by growth to confluence or by growth in isoleucine-deficient medium. They found that the S phase was the period of greatest sensitivity to all three agents and that early S appeared to be more susceptible than late S. Toxicity was also maximal during S phase, but high levels of transformation occurred when toxicity was less than 50%. These studies suggest that S phase itself may be characterized by varying susceptibility to chemical carcinogens. Similarly, Tsutsui *et al.* (7) demonstrated that early S phase is most susceptible to the induction of transformation by the combined incorporation of BrdUrd during S phase and subsequent irradiation with near-ultraviolet light. In that study, early-passage Syrian hamster embryo cells were synchronized by culture in a medium containing a low concentration of serum and hydroxyurea.

That the S phase is most susceptible to the actions of 1-β-D-arabinofuroanosylcytosine, BrdUrd, fluoro-2′-deoxyuridine, and 5-fluorouracil is not surprising because their effects are predicated upon their incorporation into DNA or upon their ability to inhibit the production of precursors required for the synthesis of DNA. MNNG, however, alkylates DNA equivalently during all phases of the cell cycle. Differences in methods used in the two previous studies that examined the effect of MNNG on toxicity and transformation in synchronized cells *in vitro* may have caused disagreement on the pattern of cycle phase sensitivity of both toxicity and transformation.

In their pioneering study, Bertram and Heidelberger (3) applied a single concentration of MNNG that allowed the survival of less than 0.1% of the exposed population of cells at all times during the cycle. Furthermore, maximal transformation frequency occurred at times of minimal toxicity, raising the possibility that periods of increased transformation were determined simply by a higher proportion of surviving cells and not by increased intracyclic sensitivity to transformation. Our data support this possibility by indicating that cell inactivation prevents the expression of many potentially transformed cells. At the high concentrations of MNNG used by Bertram and Heidelberger (3), our study shows that cell sterilization vastly predominates over transformation. This fact makes the apparent rate of transformation found at high doses depend greatly on the numbers of cells that survive in a condition allowing them to proliferate.

Although Marquardt (4) used MNNG doses that killed less than 50% of the treated population, he used a synchronization technique (thymidine-induced arrest of proliferation) that is toxic for cells and that prevents evaluation of the G_1 phase of the cell cycle (11).

Our study supplements and extends these previous reports by providing information about the effect of varying doses of MNNG on cycle dependency of toxicity and transformation in 10T1/2 cells. The results demonstrate clearly that both toxicity and transformation in synchronized 10T1/2 cells are maximal when these cells are treated during S phase. During the G_1 phase, toxicity and, to a lesser extent, transformation increase as the time of exposure to equal doses of MNNG approaches the beginning of S phase. Because alkylation of DNA is equivalent during all phases of the cycle, this observation suggests that lesions created during G_1 can be partially repaired before S phase is reached.

In a separate study we measured the removal of selected alkylation products from DNA of synchronized 10T1/2 cells (17). This study demonstrated decisively that N^7-methylguanine and O^6-methylguanine residues were excised from DNA during the G_1 phase of the cell cycle but not during the S phase. Thus, when 10T1/2 cells commence the replication of DNA during S phase, neither of these alkylated bases is removed from DNA until S phase is completed. The present studies suggest that toxicity and transformation rates are predicated on the amount of damage existing in DNA at the onset of S phase, but they also indicate that within the S phase there is a considerable variation in sensitivity to toxicity and transformation, with early S phase being most sensitive.

A similar variation in the relative susceptibility of S phase was also observed by Jones *et al.* (5, 6) and Tsutsui *et al.* (7) in studies with compounds whose action requires that they be incorporated into DNA during S phase. These similar results with different carcinogenic regimens may suggest the possibility that specific parts of the genome that replicate early in S phase may be involved in the S phase-determined predisposition to transformation. In our study, sensitivity to transformation appears to increase at about the time the first cohort of cells pass the G_1–S border and to decrease when the last cohort of cells has entered S phase. However, further studies with more precisely synchronized populations of cells will be required to determine whether sensitivity to transformation in individual

cells is correlated with the initiation of S phase, with the synthesis of a specific gene, or with some other event that occurs at or sometime after the onset of DNA synthesis.

Although the occurrence of both toxicity and transformation is maximal when MNNG is applied during the S phase, the molecular mechanisms underlying these effects seem to differ. Although synchronized 10T1/2 cells are much more likely to be inactivated by a toxic event than to be transformed by MNNG, the ratio of these two effects varies during the cell cycle. Furthermore, the relationship between dose of MNNG and each effect is also different: toxicity is directly related to dose, whereas the logarithm of transformation is directly related to dose. MNNG-induced toxicity appears to result from inactivation of relatively large amounts of the genome, preventing DNA from being replicated successfully and preventing cells from proliferating. Transformation, on the other hand, may result from more subtle lesions that allow the DNA template to be replicated, because the expression of a transformed clone as an altered focus requires repeated proliferation of the initiated cell.

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