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Cell Proliferation in Carcinogenesis

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Chemicals that induce cancer at high doses in animal bioassays often fail to fit the traditional characterization of genotoxins. Many of these nongenotoxic compounds (such as sodium saccharin) have in common the property that they increase cell proliferation in the target organ. A biologically based, computerized description of carcinogenesis was used to show that the increase in cell proliferation can account for the carcinogenicity of nongenotoxic compounds. The carcinogenic dose-response relationship for genotoxic chemicals (such as 2-acetylaminofluorene) was also due in part to increased cell proliferation. Mechanistic information is required for determination of the existence of a threshold for the proliferative (and carcinogenic) response of nongenotoxic chemicals and the estimation of risk for human exposure.

CERTAIN CHEMICALS HAVE LONG BEEN ASSOCIATED WITH cancer in humans, and animal models have been developed to study processes involved in the transition from a normal to a cancer cell (*1*). During the past two decades, emphasis has been shifting from the use of animal models primarily for the study of carcinogenic mechanisms to the use of animals to assay for carcinogenic potential of chemicals (*2*). Research has been directed more at quantitatively estimating the risk to humans. Traditionally, risk assessments have entailed the use of various mathematical and statistical formulations to extrapolate from results of high-dose animal bioassays to estimates of risk at low doses (*3*). However, high-dose tumor response data are inadequate for this purpose, as is most evident when efforts are made to predict a threshold below which there is no effect. These limitations indicate the need to base risk assessments on knowledge of the biology of tumor formation.

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We have developed a model of carcinogenesis, based on biological data and principles, that we originally used as an analytical tool to interpret results of experiments with the bladder carcinogen *N*-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide (FANFT) in rats (*4*). We demonstrated quantitatively that the tumorigenic effects of FANFT administration result from its dose-dependent genotoxic and proliferative effects, and that the proliferative effects operated only at the highest doses employed (*4, 5*).

The model can be viewed as an assembly of dynamic relationships between variables that contribute to tumor production (Fig. 1), and incorporates several biological suppositions. A fundamental assumption is that cells exist within one of three states, normal, initiated (intermediate), or transformed, and that transitions between states occur or are irreversibly fixed only in replicating cells. These transitions are assumed to take place in a stochastic fashion and represent genetic changes introduced during cell replication, possibly with the involvement of oncogenes or tumor suppressor genes (*6*). Transformed cells are those that are malignant, not cells in benign lesions. In the absence of a genotoxic exposure, the probability of a transition occurring is small but not zero (thus accounting for spontaneous tumors). The likelihood of producing a cancerous cell is increased if either the probability of a genetic transition or the rate of cell replication is increased.

Another model that also incorporates the effect of cell proliferation and was validated using human epidemiology data lends further support for a two-event hypothesis for carcinogenesis (*7*). Although based on similar biological parameters, our model uses a different mathematical construct. To represent the biological dynamics within the target organ, we resorted to a recursive simulation. Beginning with its early development period, the status of the cell population in the target organ was computed in simulated time using the probabilities for each possible event (mitosis, genetic transition, or death) facing each cell within each of a series of specific time intervals. Calculations for each subsequent time interval incorporate the results of the preceding interval. The probabilities of mitosis or death are estimated by observation of cell proliferation and cell number at various times, and the probabilities of genetic transition were inferred by a comparison of model outcomes with

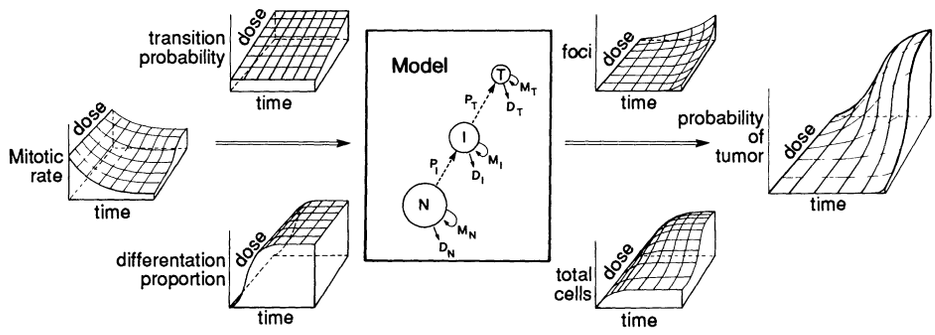

Fig. 1. A mathematical model of carcinogenesis that entails two irreversible transitions, from normal (N), to initiated (I), to transformed (T) cell populations. Population mitotic rates, M_N, M_I, and M_T, respectively, and cellular differentiation (and death) rates D_N, D_I, and D_T are primary model inputs. The interaction of these rates determines the size of cell populations. Initiation and transformation transitions occur randomly during cell replication, represented by the probabilities p_I and p_T. Model inputs are dependent on dose and animal age. Model outputs that can be validated with experimental data include target organ size (total number of cells), number of initiated cell foci (hyperplastic foci in the liver), and the probability of a visible tumor. The model is implemented computationally using stochastic simulation.

the observed time course of tumor development at the particular dose being simulated. Although this simulation approach precludes the possibility of directly estimating genetic transition probabilities and other experimentally unobservable model parameters using statistical inference, it does not risk the mathematical oversimplification required for the derivation of a computationally tractable expression that would relate tumor incidence to cellular proliferation and genetic transition variables. The quest for closed-form expressions is problematical because of the multiplicity of cellular states and the time- and dose-varying nature of the numerous cell behavior variables.

To illustrate the critical role of cell proliferation in carcinogenesis, we discuss here two prototypical compounds: a genotoxic carcinogen, 2-acetylaminofluorene (2-AAF), and a nongenotoxic agent, sodium saccharin.

2-Acetylaminofluorene (2-AAF)

To determine the tumorigenicity of 2-AAF at low doses, more than 24,000 female BALB/c mice were fed different doses (30 to 150 ppm) of 2-AAF for different periods of time (9 to 33 months) and killed at various intervals between 9 and 33 months of study (*8*). This "megamouse" experiment was designed to detect a 1% increase in the prevalence of tumors (thus is referred to as the ED_{01} study) in two target organs, liver and urinary bladder. Rather than demonstrating how to extrapolate to low doses, this study raised additional questions (*8–10*). The dose-response curve for the liver was nearly linear down to the lowest amount administered, 30 ppm. In contrast, the dose-response curve for the bladder was nonlinear. At doses below 60 ppm, there was no detectable increase in bladder tumor prevalence compared to controls, whereas prevalence increased sharply at doses above 60 ppm. Examination of tumor response as a function of time complicated the issue further (*9*).

Initially, investigators postulated that the differences in dose-response curves between liver and bladder could be explained by differences in 2-AAF toxicokinetics, and that binding of 2-AAF to DNA would not occur in the bladder below some threshold, whereas in liver even the lowest doses would have an effect. However, the administration of 2-AAF to BALB/c mice at similar and lower doses (5 to 150 ppm) produces a linear dose-response relationship for DNA adduct formation in both the liver and bladder (*11*).

The Armitage-Doll multi-stage model was also applied to explain the differences in 2-AAF response between liver and bladder tissues, leading to the postulation of a one-hit carcinogenic phenomenon for the liver and a three-hit process for the bladder (*11*). By accounting for the proliferative effects of 2-AAF in addition to its effects on DNA, which the Armitage-Doll model is unable to do, we are able to explain both dose-response curves using a two-event model of carcinogenesis (*10*).

Liver response to 2-AAF. In normal hepatocytes, 2-AAF is metabolized to its active, N-sulfated metabolite, which forms DNA adducts (*11–13*). This is reflected in our model by raising the probability of the first genetic event (p_I) above background. In contrast, cells in hyperplastic foci do not metabolize 2-AAF as readily, and considerably fewer DNA adducts are formed (*12*). Apparently, 2-AAF has a negligible effect on the probability of the second genetic event (p_T). At doses utilized in the ED_{01} study, enlargement of the liver is not observed (*8*), providing evidence of no increased hepatocyte proliferation. Thus, the only apparent impact of 2-AAF on the liver was an increase in p_I over background levels; p_T and hepatocyte mitotic rates remained at background levels and were not affected by 2-AAF administration.

Mitotic rates in the normal adult liver are relatively low (labeling index $\leq 0.1\%$). During the high proliferative phase of organ development, occasional cells are likely to become initiated, even with a low, background value for p_I. The remainder of the animal's life can then provide sufficient opportunity for at least one of these initiated cells to progress to a transformed cell, and then proliferate to a tumor of detectable size. In the ED_{01} study, spontaneous liver neoplasms were observed in 2.3% ($n = 383$) of control mice sacrificed at 24 months and 34.8% ($n = 23$) of mice sacrificed at 33 months (*8*), illustrating the influence of elapsed time on tumor development.

With a potent genotoxic compound such as 2-AAF, the relatively small number of cells initiated spontaneously during organ development is insignificant compared to the number initiated by reaction with 2-AAF metabolites (because of the increased p_I). The large number of initiated cells after exposure to 2-AAF, in combination with subsequent proliferation and transformation at background rates, results in an increased prevalence of liver tumors, particularly as the animal ages beyond 2 years (Fig. 2). At doses higher than those used in the ED_{01} study, 2-AAF also increases compensatory proliferation of surviving hepatocytes and sharply increases tumor prevalence as early as 6 months (*13*).

Bladder response to 2-AAF. Metabolism of 2-AAF in the liver also involves production of the *N*-glucuronide, which accumulates in the urine and is hydrolyzed to an electrophile that can react with both normal and initiated urothelial cells (*11, 14*). Thus, 2-AAF affects both p_I and p_T in the bladder. The relationship between 2-AAF dose and DNA adduct formation is apparently linear within the 5 to 150 ppm range (*11*). In contrast to the situation in liver, 2-AAF induces urothelial hyperplasia at doses $\geq$60 ppm (Fig. 3) (*8*). Modeling the interaction of these responses to 2-AAF effectively duplicates the in vivo results (*8, 10*) (Fig. 2). Below 60 ppm, the apparent lack of increase in tumor prevalence reflects the minimum experimental

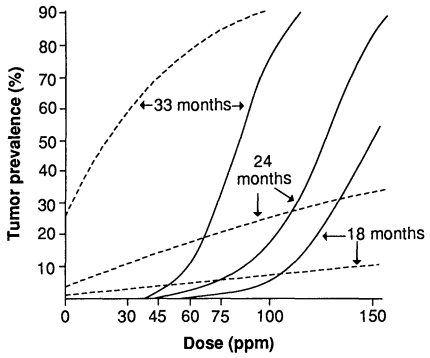

Fig. 2. Model results for effects of duration of exposure (18, 24, or 33 months) and 2-AAF dose on liver tumor (----) and bladder tumor (—) prevalence in mice. These analytical results have been demonstrated as being consistent with actual data from the ED_{01} study (*8, 10*).

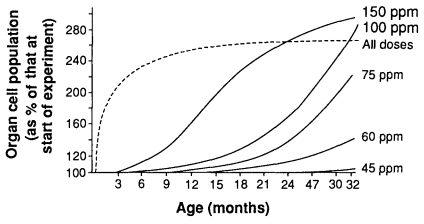

Fig. 3. Effect of normal growth, duration of exposure, and 2-AAF dose on total number of liver hepatocytes (----) and bladder urothelial cells (—) in mice. The increase in number of hepatocytes parallels the normal growth of the liver (*10*). The increase in bladder cell number caused by 2-AAF is quantified from histopathology information from the ED_{01} study (*8, 10*). 2-AAF administration began at approximately 1 month of age.

detection limit (1%) rather than the absence of tumors. At the higher doses, increased cell proliferation has an impact, and an increase in tumor formation occurs. From our modeling analyses of hypothetical situations, we calculated that if 2-AAF influenced only p_I and p_T in the bladder, tumor prevalence at 24 months would be 4% at a dose of 150 ppm, whereas, if only cell proliferative effects were present, the corresponding tumor prevalence would be 6%. The prevalence with both operating simultaneously is 88%, suggesting a synergistic effect between genotoxicity and proliferation.

Sodium Saccharin

Dietary administration of high doses of sodium saccharin (NaS) to rats over two generations results in a significant increase in the frequency of bladder cancer, particularly in males (*15, 16*). In these two-generation studies, NaS feeding begins in the dam, is continued through gestation and lactation periods, then through the lifetime of the offspring. Subsequent experiments have shown that NaS administration beginning at birth results in essentially the same tumor prevalence as with NaS administration from conception (*16*), but NaS administration started after weaning usually produces an insignificant response (*15, 16*). However, if the post-weaning rat is first treated with a short regimen of a bladder carcinogen, such as FANFT, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), or *N*-methyl-*N*-nitrosourea (MNU), followed by NaS, tumors result (*5, 17*).

Unlike 2-AAF, saccharin is nucleophilic, is not metabolized to a reactive electrophile, does not react with DNA, and is not mutagenic in most short-term assays (*17*). However when NaS is administered to the rat at high dietary doses, proliferation in the urothelium increases, resulting in mild focal hyperplasia (*17*).

Role of cell proliferation. Modeling analyses demonstrate that NaS-induced cell proliferation is sufficient to account for the increase in bladder tumor prevalence after exposure to NaS (*18*). In the FANFT-NaS experiments, tumors are produced by the stimulating effect of NaS on the dynamics of a pool of FANFT-initiated cells. Because a nonzero probability of spontaneous genetic transformation (p_T) is associated with each mitosis of an initiated cell, an increase in the mitotic rate after exposure to NaS increases the number of opportunities for transformation.

In studies where NaS administration is not preceded by initiation with a genotoxic compound, it is possible to produce an increased number of initiated cells strictly by the increase in proliferation that occurs when NaS administration is begun early in the developmental period. Because the bladder already has a maximally proliferating epithelium during gestation (labeling index approximately 10%), NaS administration during the in utero period does not further increase the proliferation rate (*17*). However, during the 3 weeks after birth, the labeling index normally declines to <0.1%. Although relatively brief, this 3-week period is of disproportionate biological importance because approximately one-third of the total number of cell divisions in a rat's 2-year life-span occur during this period (*18*). A significant increase in cell proliferation rates during the 3 weeks after birth, coupled with the background probability of spontaneous genomic errors, can substantially increase the number of initiated cells. In assessing the carcinogenicity of nongenotoxic chemicals such as NaS, it is critical to consider the increased number of initiated cells generated during fetal and neonatal development and the resulting increase in tumor prevalence to experimentally detectable levels (*17*).

An increase in the number of initiated cells caused only by excess proliferation has also been demonstrated in male rat bladders after weaning. The epithelium was ulcerated by freezing, and the resultant burst of mitotic activity was comparable to that seen during fetal development (*19*). Within 3 to 4 weeks the epithelium healed and returned to mitotic quiescence and normal morphology. Nevertheless, if high doses of NaS are subsequently administered, bladder tumors result. In terms of our model, a sufficient number of initiated cells are generated spontaneously during the regenerative hyperplasia such that the increased and sustained proliferative activity induced by NaS generates tumors (*18, 19*).

Proliferative mechanism and threshold. Utilizing traditional risk assessment methods, the results described above in male rats with extremely high doses of NaS can be extrapolated to arrive at an approximate calculated risk for humans exposed to low doses of NaS (*20*). However, there is clearly a need to understand the underlying mechanisms of carcinogenesis by nongenotoxic compounds before any rational estimate of human risk can be made. The complexity of the task in risk assessment is indicated by the finding that female rats are much less susceptible to bladder tumorigenesis in response to NaS than males, and mice, hamsters, and monkeys are resistant even at high doses (*15, 17*).

The different salt forms of saccharin produce markedly different urothelial proliferative responses (*21*). Potassium saccharin somewhat increases urothelial proliferation relative to controls, but less than does NaS. Urothelial proliferation after treatment with calcium saccharin and acid saccharin is statistically indistinguishable from controls; thus it might be assumed that neither calcium saccharin nor acid saccharin would be carcinogenic in the rat model. Absorption and urinary excretion of the saccharin anion is similar regardless of which form of saccharin is administered, but the physiological changes in the urine associated with the high loads of the different salts produce marked differences in urinary pH, ion concentrations, volume, and osmolality. The changes in pH and salt concentrations do not alter the ionic structure of saccharin, and there is no evidence that saccharin interacts directly with a urothelial cell receptor (*17*). A similar increased proliferative and tumorigenic activity in the male rat urothelium following chemical initiation is seen with high doses of several other sodium salts of weak to moderate organic acids, many of which are naturally occurring and essential for the well-being of living organisms, including vitamin C, glutamate, and

bicarbonate (*5, 17*). No tumorigenicity is observed when the acid form of these chemicals was tested (*5, 17*).

We have recently observed that, in addition to the normally present $MgNH_4PO_4$ crystals, many crystals in the urine of rats fed high doses of NaS contain silicate, and a large amount of a flocculent precipitate that contains silicate is also present (*22*). The silicate crystals and precipitate appear to act as microabrasives or cytotoxic material for urothelial cells, resulting in focal necrosis and consequent regenerative hyperplasia. Silicate precipitate and crystals require protein for their formation (*23*). Saccharin binds to urinary protein, particularly α_{2u}-globulin (*24*), thus enhancing the precipitation and crystallization that only occasionally occurs in control male rats (*25*). Urinary acidification inhibits silicate precipitation and inhibits the proliferative effects of NaS. High levels of urinary sodium and protein enhance silicate precipitation (*23*). The principal factor that appears to predispose the male rat to silicate crystal formation following NaS feeding is the presence of large quantities of normally occurring urinary protein, especially the protein specific to the male rat, α_{2u}-globulin (*24*). The female rat has much less urinary protein that the male and is less responsive to the proliferative and tumorigenic effects of NaS on the urothelium. The mouse, a species that is not responsive to saccharin even at NaS levels of 7.5% of the diet (at least three times the apparent threshold level in male rats), has low levels of urinary protein and did not form silicate crystals when fed NaS (*25*).

The multiple physical-chemical parameters in the male rat suggest that a fairly high threshold exists for NaS dose in producing silicate crystals. It is extremely unlikely that the silicate precipitates and crystals would form in humans under normal conditions of NaS ingestion, since human urine has very little protein and has less sodium than rat urine. This is consistent with the general lack of an association in humans between NaS ingestion and bladder cancer or hyperplasia (*20, 26, 27*).

Classification of Chemicals for Human Risk Assessment

The current practice is to classify chemicals as initiators, promoters, complete carcinogens, or progressing agents. In light of the demonstrated ability of compounds to increase the risk of cancer by either directly altering DNA, increasing cell proliferation, or both, distinctions blur and traditional terminology is inadequate. We feel it is useful to classify chemical carcinogens into those that interact with DNA (genotoxic) and those that do not (nongenotoxic) (Fig. 4) (*28*). Many of the latter chemicals act by increasing cell proliferation, either by direct mitogenesis of the target cell population or by cytotoxicity and consequent regenerative proliferation. Genotoxic chemicals (2-AAF and numerous others, such as diethylnitrosamine, dimethylnitrosamine, and FANFT) usually do not exhibit a threshold for the interaction with DNA, and, at higher doses, may cause cell death resulting in cell proliferation (*5, 29*). This dual effect of genotoxic chemicals frequently leads to a dose-response curve similar to that of 2-AAF in the bladder described above. A modest rate of increase in tumor prevalence at low doses is due only to a genotoxic effect, and a much greater rate of increase at higher doses is due to the synergistic influence of increased cell proliferation. The actual dose- and time-response for a chemical is dependent on whether the compound has a genotoxic effect, a proliferative effect, or both, and whether it affects normal or initiated cells, or both.

The nongenotoxic chemicals can be further categorized by their mechanisms of action, if known. For example, phorbol esters, dioxin, and hormones each interact with a cellular receptor (*30*), whereas NaS (*17*), antioxidants (*31*), thin films, hepatotoxins, and nephrotoxins (*28*) act through a non-receptor mechanism. Cytotoxicity, direct mitogenesis, or both can also occur with chemicals acting through cell receptors (such as the phorbol esters) (*28, 30*). Compounds acting through specific receptors tend to be active at low doses, and it is unclear whther a no-effect threshold could be ascertained for these compounds. Similarly, chemicals that are directly mitogenic to target cells may or may not have a threshold. In contrast, most, if not all, compounds that act solely through a cytotoxic mechanism would be expected to have a no-effect threshold above which cytotoxicity becomes apparent. Below the threshold, cytotoxicity and increased cell proliferation would not occur, and there would be no increased risk of tumors. Interpretation of long-term bioassays for nongenotoxic chemicals must take into account aspects of nonreceptor mechanisms.

Examples of a dose-response threshold occur with uracil and melamine (*32*). If sufficiently high doses of either of these nongenotoxic chemicals are fed to rats or mice, urinary calculi, urothelial proliferation, and tumors occur. If the dose is below the minimum at which calculi occur, there is no increased cell proliferation or tumor formation.

Cell Proliferation as a Predictor of Carcinogenesis

Despite the importance of cell proliferation in carcinogenesis, short-term assays of increased cell proliferation in response to nongenotoxic chemicals are likely to prove as inadequate as short-term genotoxicity assays for predicting carcinogenicity. Some chemicals induce only a temporary or mild increase in proliferation that may not be adequate to produce a detectable increase in tumor prevalence within the lifetime of the experimental animal. Also, increased proliferation must occur in cells susceptible to cancer development, rather than in nonsusceptible cells, such as terminally differentiated cells, that may also be present in the target organ. For example, turpentine can cause proliferation of the skin, but is a very weak skin tumor promoter (*33*). Turpentine primarily increases proliferation of the keratinocytes rather than the dark basal cells that are the apparent precursors of skin tumors.

Confusion can also arise with chemicals such as cyclophosphamide (*34*). Although it is extremely cytotoxic to the bladder epithelium, leading to a marked regenerative hyperplasia, it is also

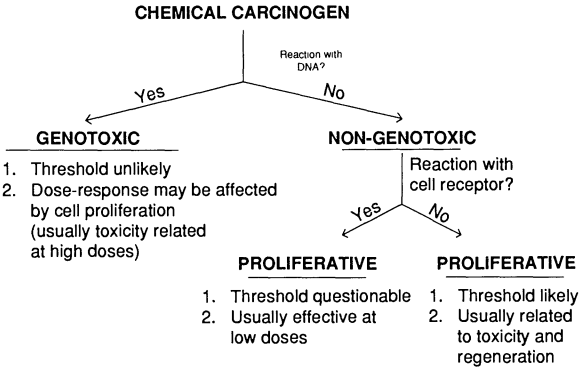

Fig. 4. Proposed classification scheme for carcinogens. The effect of genotoxic chemicals can be accentuated if cell proliferative effects are also present. Nongenotoxic chemicals act by increasing cell proliferation directly or indirectly, either through interaction with a specific cell receptor or nonspecifically by (i) a direct mitogenic stimulus; (ii) causing toxicity with consequent regeneration; or (iii) interrupting physiological process. Examples of the latter mechanism include TSH stimulation of thyroid cell proliferation after toxic damage to the thyroid, and viral stimulation of proliferation after immunosuppression.

cytotoxic to any bladder tumor cells that might form. If cyclophosphamide is administered at doses high enough to be genotoxic but below those that are cytotoxic, the prevalence of bladder tumors is increased in animals and humans. At higher cytotoxic doses, regenerative hyperplasia occurs but no tumors are produced.

There are numerous indications in humans that prolonged, increased cell proliferation is necessary for the development of tumors, particularly for hormonally related tumors such as estrogen-related endometrial carcinomas (*35*). It appears that most virally related human tumors are also a result of sustained increased proliferation. For example, Epstein-Barr virus (EBV) stimulates B lymphocyte proliferation. When a patient is immunosuppressed, whether due to heredity, immunosuppressive drugs associated with transplantation, or AIDS, the B-cell proliferation cannot be controlled, and there is an appreciable increase in the risk of B-cell lymphomas (*36*). Hepatitis B virus (HBV) can produce chronic hepatitis and cirrhosis, characterized by persistent necrosis and regenerative hyperplasia, and is also associated with an increased incidence of hepatoma (*37*).

It would appear that increased cell proliferation also contributes to the development of tumors secondary to various chemical exposures in humans. For example, cigarette smoking is known to cause bladder cancer in humans, perhaps due to a hyperplastic effect on the urothelium of many cigarette smokers, in addition to the probable genotoxic damage that occurs (*27*).

As the mechanisms of carcinogenesis become more thoroughly understood, a more rational approach can be taken for extrapolation from high dose experimental data in animals to low dose natural exposure and assessment of the risk faced by human populations exposed to chemical agents. The effects of toxicity and consequent cell proliferation are particularly critical for nongenotoxic agents, because a threshold effect is likely.

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