Environmental Health Perspectives
Vol. 76, pp. 57-63, 1987

High- to Low-Dose Extrapolation: Critical Determinants Involved in the Dose Response of Carcinogenic Substances

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Recent investigations on mechanisms of carcinogenesis have demonstrated important quantitative relationships between the induction of neoplasia, the molecular dose of promutagenic DNA adducts and their efficiency for causing base-pair mismatch, and the extent of cell proliferation in target organ. These factors are involved in the multistage process of carcinogenesis, including initiation, promotion, and progression. The molecular dose of DNA adducts can exhibit supralinear, linear, or sublinear relationships to external dose due to differences in absorption, biotransformation, and DNA repair at high versus low doses. In contrast, increased cell proliferation is a common phenomena that is associated with exposures to relatively high doses of toxic chemicals. As such, it enhances the carcinogenic response at high doses, but has little effect at low doses. Since data on cell proliferation can be obtained for any exposure scenario and molecular dosimetry studies are beginning to emerge on selected chemical carcinogens, methods are needed so that these critical factors can be utilized in extrapolation from high to low doses and across species. The use of such information may provide a scientific basis for quantitative risk assesesment.

Introduction

Tumor incidence data from chronic bioassays on many important industrial and environmental chemicals are increasingly being factored into quantitative assessments of potential cancer risk. These estimates of risk are usually for human exposures that are orders of magnitude lower than those used in the animal studies. For such estimates to be accurate, one must first identify which factors are critical determinants in the dose-response relationship for carcinogenesis, and then appropriately weigh those factors that change between high and low exposure.

Recently, considerable attention has focused on the potential of using "molecular dose" as one such factor (*1–3*). The molecular dose is the amount of an agent that reaches a critical target site, usually a macromolecule such as DNA or hemoglobin. The actual entity that is quantitated (i.e., DNA adduct) is the molecular dosimeter, and the process of measuring the molecular dose is termed "molecular dosimetry." Since many factors such as absorption, distribution, and biotransformation represent saturable processes, it is probable that the molecular dose will bear a closer relationship to biological effects than would external exposure. If metabolic activation becomes saturated, as is the case for vinyl chloride (*4*) and NNK (*5–7*), then exposures above the saturation point would result in molecular doses lower than predicted. On the other hand, if detoxification becomes saturated at high exposure, the molecular dose would be higher than would be predicted from the low exposure data. Thus, bioactivation and detoxification are critical determinants in extrapolation from high to low doses.

Since cancer is believed to involve heritable changes and DNA is the material that carries heritable information, DNA adducts have been closely studied as potential molecular dosimeters for genotoxic carcinogens. Unfortunately, many carcinogens react at more than one site on DNA, producing several types of DNA adducts. Therefore, it becomes important to consider all available biological data, including rate of formation, repair, and mutagenic efficiency, when choosing the most appropriate DNA adducts to be used as molecular dosimeters. For example, alkylating agents modify DNA at the ring nitrogens and exocyclic nitrogens and oxygens of pyrimidine and purine bases. Adducts resulting from alkylation at ring nitrogens (i.e., N-7-deoxyguanosine and N-3-deoxyadenosine) show little

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FIGURE 1. Quantitative factors that are involved in the mutational events of initiation include the number and efficiency of DNA adducts for causing mutations when cell replication occurs prior to DNA repair. Rapidly proliferating cells have less time between successive cell divisions for DNA repair and are therefore more likely to be initiated.

correlation with tumor formation (*8*). In contrast, a large body of evidence has demonstrated that alkylation of the exocyclic oxygens, especially O^6-alkyldeoxyguanosine (O^6-alkyldG) (*8–10*) and O^4-alkyldeoxythymidine (O^4-alkyldT) (*11,12*), does correlate with tumorigenesis, mutagenesis, and the ability to mispair during replication (*13,14*). Thus, in the case of alkylating agents, it may be necessary to monitor more than one DNA adduct, since several adducts could be contributing to the carcinogenic process. Simple alkylating agents may represent the most complex carcinogens with respect to the many types of DNA adducts they induce. Carcinogens such as 2-acetylaminofluorene and the polycyclic aromatic hydrocarbons form only a few predominant adducts. For these types of carcinogens the complex decision of choosing an appropriate dosimeter may be greatly simplified.

Choice of a molecular dosimeter should also take into consideration the exposure regimen. Pulse dose and *in vitro* experiments indicate DNA adducts that may be mechanistically involved in the induction of cancer by correlating their persistence with the carcinogenicity of the chemical and by measuring the extent to which they interfere with the fidelity of DNA replication. However, these experiments may not accurately portray the kinetics of formation and repair that operate during chronic exposure regimens such as those used in most rodent bioassays or encountered during environmental exposures.

In addition to DNA adduct formation related to known chemical exposure, DNA damage can occur from unknown exposures such as irradiation and contaminants in the diet, air, or water. Presently, such spontaneous DNA damage cannot be quantified, but it is likely to play a role in spontaneous neoplasia, such as occurs in unexposed control animals (*15*). Spontaneous DNA damage may also be involved in carcinogenic responses of nongenotoxic carcinogens.

As mentioned above, different DNA adducts have different efficiencies for causing mispairing and therefore different probabilities for inducing mutations. Under ideal circumstances such data would be known for each DNA adduct present in a tissue. Thus, the quantity of each molecular dosimeter and its efficiency for causing mutations could be utilized in quantitative risk assessment. It is clear, however, that this alone will not be adequate. Cell replication is an essential component of every stage of the carcinogenic process. Mechanistically, replication of DNA containing carcinogen-induced or spontaneously occurring adducts produces mutations in the daughter strand (Fig. 1). When these mutations occur at critical sites in the genome, they are part of the irreversible and heritable changes involved in the initiation process of chemical carcinogenesis. Promotion

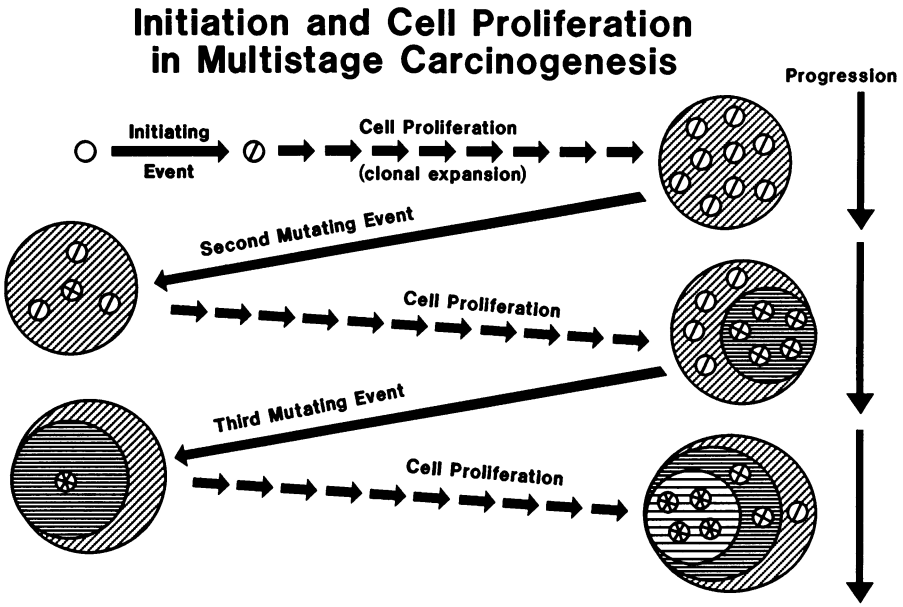

FIGURE 2. Schematic of the role of cell proliferation in the initiation, promotion, and progression of carcinogenesis. The extent of clonal expansion necessary for second or third mutational events is dependent on the amount of spontaneous and chemically induced DNA damage.

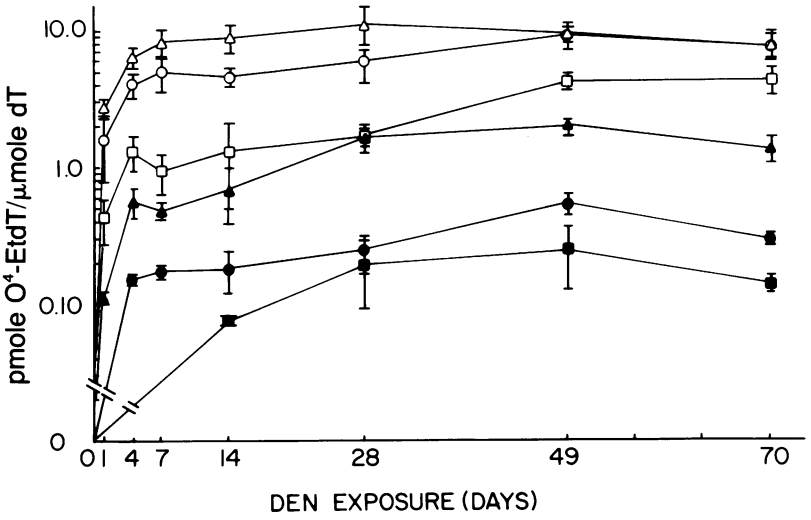

FIGURE 3. Accumulation of O^4-EtdT during continuous exposure of rats to 0.4 (■), 1 (●), 4 (▲), 10 (□), 40 (O), or 100 (△) ppm DEN. Adduct concentrations are expressed versus time of exposure, 1, 4, 7, 14, 28, 49, or 70 days. Note the log scale of the y axis. Bars, mean ± SE for three to four animals. The O^4-EtdT concentrations were below the limit of detection of the radioimmunoassay (< 0.2 pmole O^4-EtdT/μmole dT) for exposure of 0.4 ppm DEN for 1, 4, and 7 days and 1 ppm DEN for 1 day. From Boucheron (*20*).

has been described as cell proliferation leading to the selective clonal expansion of the initiated cell population (Fig. 2). This expansion increases the probability of further genetic alterations in the initiated cell population believed to be necessary in the multistage process of carcinogenesis (*16,17*). In the final stage of carcinogenesis, cell proliferation is essential for the irreversible progression from benign focal proliferations to malignant neoplasms (*16*).

Cell replication is frequently increased in tissues exposed to toxic doses of a chemical. Thus, the extent of cell proliferation can vary greatly between high and low exposures. Since cell proliferation is clearly involved in carcinogenesis, such dose-responsive changes need to be factored into the dose response for carcinogenesis.

The best data for extrapolating the extent of initiation from high to low exposures should be provided by the combination of molecular dose, coupled with efficiency for mispairing for each molecular dosimeter and the extent of cell proliferation over the range of exposure from that tested in animals to that expected for humans (Fig. 1). Cell proliferation also should modulate clonal expansion and second mutational events in a dose-responsive manner. While we do not have a complete understanding of these interactions for a single chemical, data covering one to three orders of magnitude of exposure are becoming available for several carcinogens on the molecular dosimetry of major DNA adducts and the extent of cell proliferation. Examples of these data are described in the following sections.

Diethylnitrosamine

Diethylnitrosamine (DEN) represents one of the best-studied chemical carcinogens. Chronic bioassay data, complete with detailed analyses of tumor incidence and survival, are available covering exposures over three orders of magnitude (*18*). Research on the formation and repair of DNA adducts induced by DEN have shown that O^4-EtdT is the major promutagenic adduct in liver DNA of chronically exposed rats (*11*). Although O^6-EtdG is chemically formed at concentrations three- to fourfold greater than O^4-EtdT, it is efficiently removed by the O^6-alkylguanine-DNA alkyltransferase. In contrast, O^4-EtdT is repaired slowly, having a $t_{1/2}$ of approximately 11 days (*19*). This leads to concentrations of O^4-EtdT that are approximately 50 times higher than O^6-EtdG.

More recently, experiments on the molecular dosimetry of O^4-EtdT have been conducted using rats exposed to 0, 0.4, 1, 4, 10, 40, or 100 ppm DEN in the drinking water for up to 70 days (*20*). As shown in Figure 3, O^4-EtdT accumulated in liver DNA in a dose- and time-dependent manner. All exposure groups had O^4-EtdT concentrations that reached apparent steady-state. The time to steady-state appeared to be dose-dependent. The two higher exposures attained nearly 90% of steady-state concentrations by 7 days; lower exposures took somewhat longer. The apparent steady-state concentrations of O^4-EtdT were relatively linear with respect to dose from 0.4 to 40 ppm. Concentrations at the highest exposure (100 ppm) were lower than predicted by linear extrapolation.

Cell replication also increased in a dose- and time-dependent manner at higher DEN concentrations (Fig. 4). However, the numbers of labeled hepatocytes in rats exposed to 1 or 0.4 ppm DEN were not significantly greater than those of controls, indicating that increased cell replication is primarily associated with exposure to high doses of DEN. Labeling indices were increased as

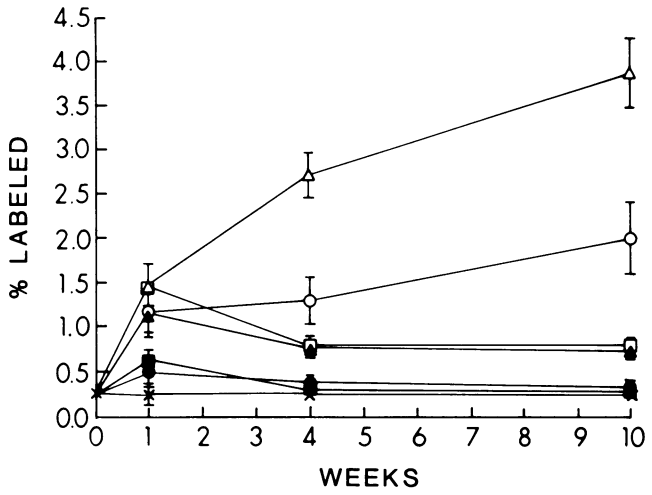

FIGURE 4. The percent of hepatocytes labeled with [^{3}H]thymidine during exposure to 0(X), 0.4(■), 1(●), 4(▲), 10(□), 40(○), or 100(△) ppm DEN.

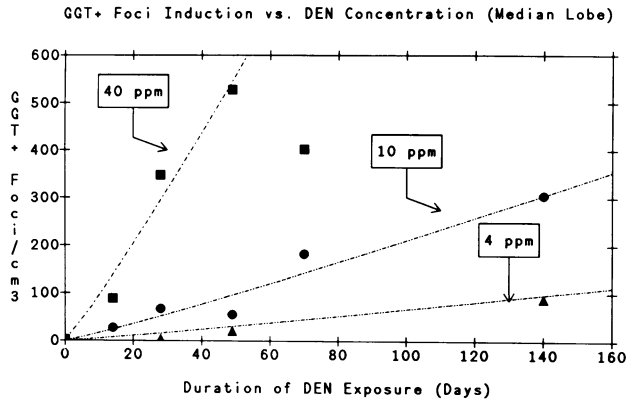

FIGURE 5. Induction of GGT+ foci in the right median hepatic lobe during continuous administration of DEN.

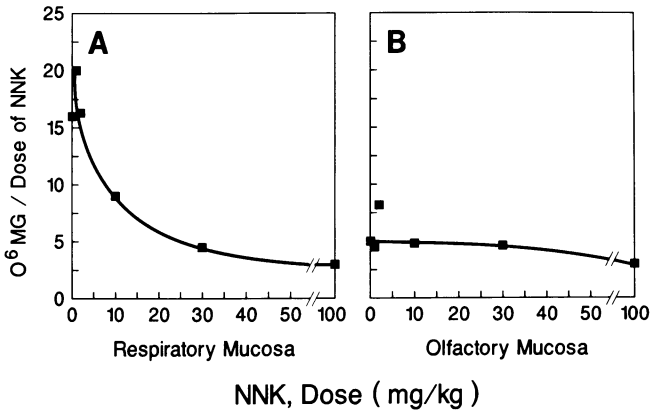

FIGURE 6. Concentration of O^6-methylguanine in respiratory and olfactory mucosa of DNA of rats exposed to NNK. The data have been normalized per unit dose of NNK and demonstrate that low doses of NNK are more efficient than high doses in producing O^6-methylguanine in the respiratory portion of the nasal passages. From Belinsky et al. (*5*).

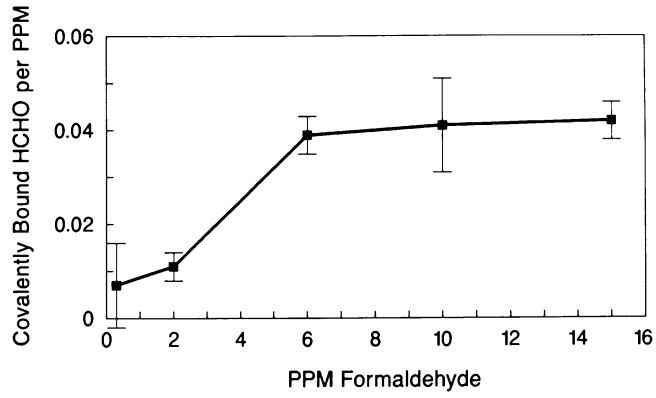

FIGURE 7. Concentration of covalently bound formaldehyde in respiratory mucosal DNA normalized per part per million of inhaled formaldehyde. Formaldehyde is less efficient in producing covalent binding at low versus high exposures. Data calculated from Casanova-Schmitz et al. (*23*).

much as 15 times that of controls in the left lobes of rats exposed to 100 ppm DEN and averaged a threefold increase in animals exposed to 10 or 4 ppm DEN.

The interaction of promutagenic DNA adducts, such as O^4-EtdT, and cell replication in carcinogenesis is supported by data on hepatocyte initiation using the same DEN exposure conditions. Hepatocyte initiation showed similar trends, with the number of growth-selected γ-glutamyltransferase positive (GGT+) foci increasing with both dose and time. At 40 ppm, the highest DEN concentration studied, hepatocyte initiation increased rapidly and then plateaued (*12,21*). At 10 and 4 ppm DEN, hepatocyte initiation appeared to be dependent on the product of DEN concentration and time of exposure and was unaffected by the rate of DEN consumption (Fig. 5) (*21*). These data are consistent with data on O^4-EtdT and cell proliferation. At 40 ppm, there was a marked increase in cell proliferation (approximately ninefold), whereas at 10 and 4 ppm there was a three- to fourfold increase. Concentrations of O^4-EtdT were linear with the concentration of DEN in drinking water at all three exposures. The plateau in hepatocyte initiation is believed to represent a steady-state of initiated hepatocytes, whereby the number of newly initiated hepatocytes equals the number of previously initiated hepatocytes that are killed due to cytotoxicity. The average focus in animals exposed to 40 ppm also had a greater volume after 4 weeks of exposure than did foci induced by exposure to 10 and 4 ppm, suggesting additional selection pressure due to the increase in cell proliferation. Unfortunately, this study suggests that growth-selected GGT+ foci will not be useful short-term indicators of initiation in investigations at lower DEN exposures, since an extrapolation to the carcinogenic concentration of 1 ppm DEN predicts that it would take nearly 80 weeks to produce 100

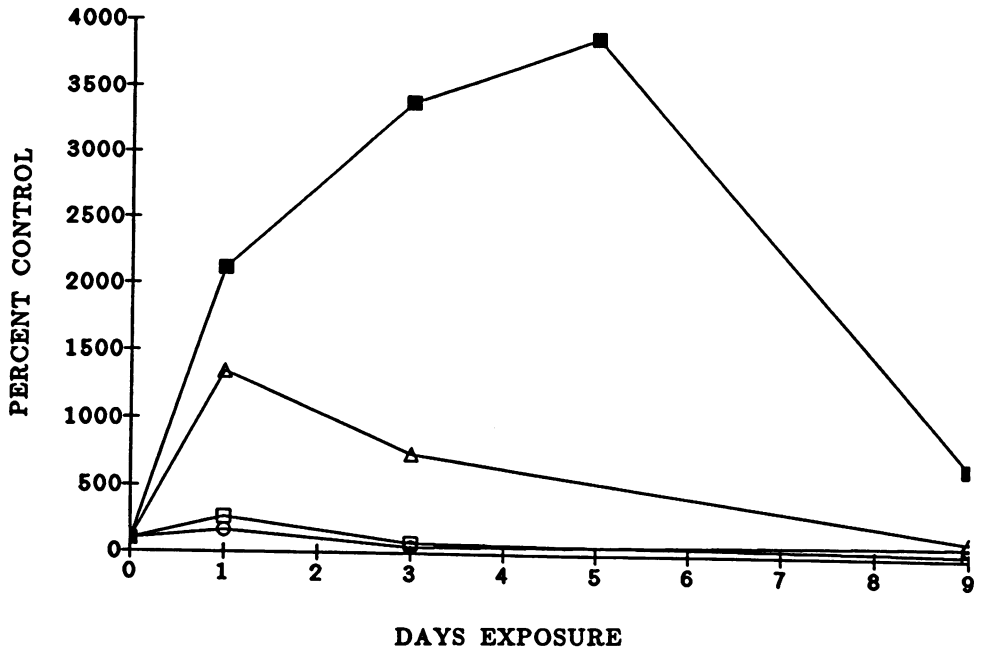

FIGURE 8. Compilation of all rat cell turnover data using the 18-hr [^{3}H]thymidine pulse. Exposures were 0.5 (□), 2 (O), and 6 (△) ppm formaldehyde 6 hr/day and the combined data from 12 ppm 3 hr/day and 15 ppm 6 hr/day (■). From Swenberg et al. (*24*).

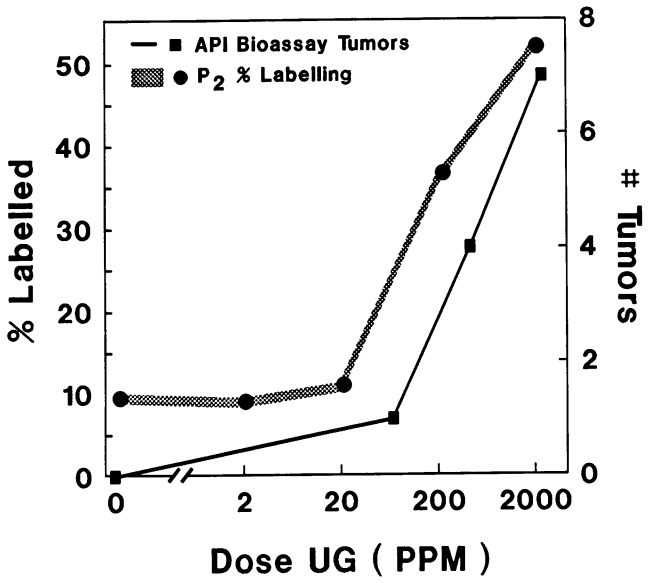

FIGURE 9. Dose-dependent similarity of P2 cell labeling index observed in male rat kidney during 3 weeks of inhalation exposure to unleaded gasoline and the number of renal epithelial tumors found in male rats exposed to unleaded gasoline for 2 years. From Short et al. (*28*).

GGT+ foci/cm^3. Even this may be an overestimate as cell proliferation was not increased at 1 ppm DEN.

NNK

Although the molecular dose of O^4-EtdT was nearly linear over DEN concentrations covering 2.5 orders of magnitude, a different phenomena was evident for O^6-MedG in rats exposed to NNK (*6*). Both lung and nasal mucosa exhibited supralinear dose-response curves for O^6-MedG, i.e., NNK induced more O^6-MedG per unit dose at low, rather than at high exposures (Fig. 6). This is believed to be due to the presence of a low K_m pathway for metabolic activation that becomes saturated at higher exposures (*5–7*).

It also appears that cytotoxicity-related cell proliferation plays a pivotal role in the induction of malignant nasal tumors (*5*). Benign tumors occur in both respiratory and olfactory mucosa; malignant tumors primarily occur in olfactory mucosa. The location of malignant tumors parallels the location of NNK-induced cytotoxicity and cell proliferation. In the absence of cytotoxicity, nasal epithelium has a very low amount of cell proliferation. This low cell proliferation is postulated to decrease the probability of a mutation due to base mispairing and to decrease the extent of clonal expansion of initiated cells.

Formaldehyde

The opposite scenario exists for formaldehyde. Molecular dosimetry experiments by Heck and co-workers have demonstrated a sublinear dose-response for DNA-protein cross-links (*22,23*). Formaldehyde exposures of 6 ppm or greater result in disproportionately more DNA-protein cross-links per part per million formaldehyde than occur at concentrations of 4 ppm or less (Fig. 7). This is the result of saturation of glutathione-dependent pathways for detoxification. Of great interest is the fact that a major nonlinearity in formaldehyde-induced nasal cancer occurs between 6 and 15 ppm, even though DNA-protein cross-links are linearly related to

external exposure over these concentrations. Again, marked increases in cell proliferation at 15 ppm formaldehyde offer the best explanation (Fig. 8) (*24*).

Unleaded Gasoline

Chronic exposure to unleaded gasoline induces kidney tumors in male rats, but not female rats or either sex of mice. This carcinogenic response is believed to result from the reversible binding of components of gasoline or their metabolites to α_{2u}-globulin, a low molecular weight protein found in the kidney of male, but not female rats (*25–27*). This binding is thought to alter the protein's conformation, making it less digestible. The resultant protein overload of the P2 segment epithelial cells causes cell death and a sustained increase in cell proliferation (*26,28*) that exhibits a dose-response similar to that of renal tumor induction (Fig. 9). Previous studies have demonstrated a lack of genotoxicity for the same sample of unleaded gasoline (*29–31*). Thus, it is likely that the increase in renal cancer in male rats represents promotion of spontaneously initiated renal epithelial cells that is secondary to a sex- and species-specific cytotoxicity. Preliminary data from a large initiation-promotion experiment support this interpretation.

Conclusions

Data on this divergent group of chemical carcinogens clearly demonstrate that several parameters represent critical facets in understanding the scientific basis for dose-response in carcinogenesis. Two critical determinants in the dose response of carcinogenesis are cell proliferation and the molecular dose of DNA adducts. Cell proliferation data can be obtained for any exposure regimen desired. As such, cell proliferation represents an important type of data that can be factored into the risk assessment process. Based on earlier studies of Moolgavkar (*32*), preliminary models employing such data are under development in several laboratories. Likewise, efforts are underway to use data on DNA adducts in place of external exposure (*2*). It is obvious from the data presented that a generic approach that assumes equal effects per unit dose between high and low exposure is almost certain to be incorrect. Thus, considerable effort and support must be given to this emerging field, if quantitative risk assessment is to be based on a firm scientific foundation.

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