

Opportunities for Improving Techniques for Interspecies Extrapolation in the Risk Assessment Process

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Quantitative estimates of human carcinogenic risk from chemical exposure are currently derived primarily from linearized multistage model analyses of the tumor response as observed in chronic laboratory animal bioassays versus administered dose. The numerous ad hoc assumptions that provide a rationale for this generic approach to carcinogenic risk assessment can only be evaluated critically when mechanistic data directly relevant to the low-dose and interspecies extrapolation problems are available. Clear needs exist to develop such ancillary data bases and the means for explicitly incorporating them into the risk estimation process. Target site dosimetry provides one useful organizing concept. Physiological response modeling can account systematically for interspecies variations in the distribution and disposition of chemicals in relation to external measures of exposure. Direct measurements of interactions of chemicals and their metabolites with specific target macromolecules can provide sensitive and biologically meaningful exposure indices. Alternatively, quantitation of toxic effects such as altered cell regulation and differentiation can serve the same purpose. Virus and oncogene activation, DNA damage and repair, and enhanced cell proliferation provide additional biological markers of exposure. They may also comprise critical elements of the carcinogenic process. Identification of the actual mechanisms involved should eventually lead to the development of risk assessment models that adequately reflect the unique biological and toxicological characteristics of different species-chemical combinations.

Introduction

At present, regulatory agencies rely primarily on linearized, multistage model analyses of the tumor responses observed in chronic laboratory animal bioassays to generate quantitative estimates of human cancer risk from any chemical exposure. This generic approach to carcinogenic risk assessment rests on numerous ad hoc assumptions (1) that are made in the absence of detailed biological and toxicological data regarding the actual mechanisms of action of chemicals. For example, it is assumed that the administered dose of a chemical is directly proportional to the amount or concentration of the carcinogenically active form of the chemical that reaches specific target tissue macromolecules. In addition, after a simple scaling of exposure levels between test animals and humans on a body weight or surface area basis, it is assumed that humans may be at least as sensitive to the carcinogenic effects of chemicals as is the most sensitive animal species.

Although it may be anticipated that assumptions of such global generality will be incorrect in many specific cases (2), these assumptions can only be evaluated critically and replaced as necessary with mechanistic data that shed light on the many complex and interacting factors that

make the interspecies extrapolation problem so difficult. The challenge is to develop detailed ancillary data bases for chemicals that not only reveal their mechanisms of action, but also permit a scientifically defensible explanation of specific and possibly discrepant study findings in mechanistic terms. As is clearly illustrated in the following examples drawn from the Chemical Industry Institute of Toxicology (CIIT) research program of methanol, 1,3-butadiene, unleaded gasoline, and formaldehyde, this approach is likely to be most fruitful where bioassay and/or shorter term study results indicate significant differences in toxic responses to chemical exposure at different dose levels, across sexes, and/or between species.

Methanol

Methanol is a major industrial chemical with a current annual production in the United States of over 1 billion gallons. While the potential for human exposure is considerable, virtually nothing is known regarding toxic effects of chronic methanol exposure. It has long been known that acute methanol exposure in humans, primarily by ingestion, may lead to metabolic acidosis, central nervous system depression, blindness, and death. Research studies designed to characterize animal models for acute, high-dose methanol toxicity have revealed that the metabolism of methanol differs signifi-

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cantly across species (3,4). Primates and rodents differ in the enzyme responsible for the initial step of methanol metabolism. Methanol is oxidized to formaldehyde almost exclusively by alcohol dehydrogenase in primates, with catalase playing little or no role. In contrast, the catalase-peroxidative system is primarily responsible for the first step in methanol oxidation in rats (5).

Methanol exposure also results in marked elevation of blood formic acid in humans and monkeys, but no formate accumulation occurs in normal rats (5). The rate for formate oxidation to CO₂ in rats is twice that in monkeys (6), and in both species, the metabolism is dependent on folic acid (6,7). Rats deficient in folic acid exhibit metabolic acidosis and formic acid accumulation when treated with methanol (8). These findings suggest that the rate of formate oxidation and the folic acid status are important determinants of the relative sensitivity of different species to acute methanol toxicity.

These studies provide valuable information on acute, high-dose methanol toxicity. However, the extreme doses and routes of administration (oral gavage or IP injection) that were used would call into serious question the validity of any extrapolations based on the resulting data to human environmental and occupational exposure conditions, where exposure occurs by inhalation of low, airborne concentrations. The current threshold limit value (TLV) is 200 ppm.

The choice of a particular animal species as a model for humans is always an important issue in the design of chronic toxicity and oncogenicity studies. Usually very little is known regarding the differences in toxicokinetics between animal species and humans, but this is not the case with methanol. Much is known regarding the difference in toxicokinetics between primates and rodents after high oral or parenteral doses and that knowledge suggests that the rat may be an inappropriate model for humans. This would seem to imply that safety evaluations of methanol be performed with primates rather than with the usual rodent species. On the other hand, the judgment that rodents are an inappropriate model is based on the lack of accumulation of formate after exposure to large methanol doses. Although formate has been strongly implicated in the acidosis and ocular toxicity that arise in primates following acute exposure, there is no guarantee that chronic exposure to low doses of methanol would produce sufficient blood concentrations of formate to cause ocular toxicity or that formate *per se* would be responsible for the toxic responses, if any, that result from chronic low-dose exposure.

Given the expense of performing any chronic toxicity study and the additional costs when primates are studied, it seems prudent to base the choice of an animal model on objective evidence to the maximum extent possible. Useful input for this choice can be derived from comparisons of methanol pharmacokinetics among rodents, subhuman primates, and humans following short-term exposures by inhalation. In particular, proper design of such studies using rodents and subhuman primates could make it possible to develop a physiologically based pharmacokinetic model for methanol of the kind

employed by Andersen and his co-workers (9,10) in their studies of styrene and other chemicals. A mechanistic distribution and disposition model of this kind could be scaled-up on a purely scientific basis using data specific to methanol and the relevant species to predict the blood concentrations of methanol or formate expected in humans, even under conditions of exposure that are not ethically attainable in direct tests. It may be possible to use such a model to identify conditions of exposure for rodents that, by virtue of adjusted temporal patterns of exposure and/or airborne concentrations, would produce blood concentrations of methanol and formate in the rodent that mirror those predicted to occur in primates and humans at or above the current TLV. Selection of dose levels for chronic studies on this basis would provide a unique way to avoid problems associated with the use of maximum tolerated doses for rodents that may have little or no relevance to human exposure conditions.

1,3-Butadiene

The potential for occupational exposures to 1,3-butadiene exists in the synthetic rubber industry where this colorless gas is employed as a comonomer in the production process. Several retrospective mortality studies of workers in this industry have indicated significantly less overall cancer mortality than occurs in the general population; however, these studies also revealed a possible association between 1,3-butadiene exposure and increased risk of death from lymphatic and hematopoietic neoplasms (11-13).

The findings of two recent bioassays have demonstrated that 1,3-butadiene is a rodent carcinogen, but some striking species differences in toxicity were observed. Sprague-Dawley rats exposed to relatively high concentrations of 1,3-butadiene (1000-8000 ppm) exhibited significant increases in a variety of solid tissue tumors, including mammary, thyroid, zymbal, and pancreatic, as well as uterine and testicular neoplasms, some of which were observed only in the highest dose group (14). In sharp contrast, exposure of B6C3F1 mice to 625 or 1250 ppm resulted in a significant elevation in the incidence of hemangiosarcomas, bronchial adenomas and carcinomas, papillomas of the forestomach, mammary adenomas, and ovarian tumors. The incidence of malignant lymphoma in mice, apparently of thymic origin, exceeded 50% in the males exposed to the highest concentration and forced termination of the study after 60 to 61 weeks of study for both dose groups (15).

The potential association of 1,3-butadiene exposure and lymphoma/leukemia in man has heightened concern regarding the occurrence of lymphoma in the treated B6C3F1 mice. However, it is currently not at all clear whether the mouse or the rat is the most appropriate animal model for assessing human risk from butadiene exposure. Studies of the mechanisms by which chemical exposures induce lymphomas in laboratory mice have been hampered by the ubiquitous presence of endogenous type C retroviruses (MuLV) that are invariably

associated with spontaneous lymphoma and leukemia in these animals. Consequently, it has not yet been possible to determine the respective contributions of chemical exposure and the endogenous retrovirus to murine leukemogenesis. Strain variations in MuLV expression and the spontaneous incidence of leukemia/lymphoma are both large, and it is clear that the presence of the endogenous virus *per se* does not produce the disease but is a critical part of a multifactorial process. Thus, cross-species differences in retroviral background could have an important bearing on butadiene toxicity and the evaluation of potential risk to humans.

Type C retroviruses have been identified as the etiologic agents in a majority of spontaneous leukemias occurring in many animal species (16). In addition, advances in immunobiology and molecular biology have permitted the isolation and definitive characterization of a human type C retrovirus, which is involved in the etiology of adult T-cell lymphomas and leukemias and has been demonstrated to transform human cells in culture (17-22). Although a large majority of individuals can be shown to express the virus (greater than 87% in one study), not all individuals who express the virus incur the disease. This strongly suggests that external factors, possibly of environmental origin, also play a role in the expression of lymphoma in these individuals (23,24).

A thorough evaluation of the mouse bioassay data requires an investigation into the possibility of indirect mechanisms of action such as viral activation that may be induced by toxic effects in target organs other than the thymus. For example, bone marrow injury is known to play an essential role in the pathogenesis of radiation-induced murine leukemogenesis (25-27). It has also been implicated in certain chemically induced murine lymphomas (28).

Studies in progress at CIIT have begun to shed light on the mechanisms underlying butadiene leukemogenesis. Thymic lymphoma was found to be the major cause of death in B6C3F1 mice chronically exposed to 1,3-butadiene at an airborne concentration of 1250 ppm. This study confirmed the previous findings of Huff et al. (15). These lesions were encountered as early as 25 weeks after the initiation of exposure. The lymphomas were of T-cell origin and expressed significantly increased levels of MuLV (29). Furthermore, the bone marrow has been identified as an important target for 1,3-butadiene toxicity in these mice. This is consistent with the results of previous studies of radiation-induced murine thymic lymphoma that indicated that bone marrow damage is an essential prerequisite for lymphoma development. The specific nature of the bone marrow damage is manifested by a macrocytic-megaloblastic anemia, cytogenetic abnormalities, and alterations in hematopoietic stem cell development (30).

The question of whether or not endogenous retrovirus expression is essential in the development of lymphoma in butadiene-exposed mice can be addressed by exploiting the marked strain variations in retrovirus expression noted earlier. Unlike the B6C3F1 mouse, NIH Swiss mice possess a unique, proviral background in

which MuLV gene sequences are truncated (31-33). Consequently, endogenous ecotropic MuLV virus has never been detected in this strain. Comparative studies of 1,3-butadiene-exposed NIH Swiss and B6C3F1 mice can provide a presumptive answer to this important question. Findings from the first of a series of studies in progress at CIIT have revealed that 1,3-butadiene exposure induces the same megaloblastic anemia bone marrow damage in NIH Swiss mice as was observed in the B6C3F1 mouse (34). These studies indicate that the bone marrow is an important murine target organ for 1,3-butadiene toxicity, irrespective of MuLV expression. Ironically, more precise knowledge of the mechanism of butadiene oncogenesis could lead either to complete invalidation of the murine lymphoma model for human risk assessment purposes or, alternatively, to the identification of specific human subpopulations that may be particularly sensitive to the effects of exposure.

Unleaded Gasoline

A 2-year inhalation study of gasoline toxicity in rats and mice revealed a significant increase in tumors of the kidney, but only among the male rats (35,36). Dose-dependent nephrotoxicity, including the appearance of hyaline droplets (abnormal amounts of intracellular protein) in the proximal portions of the kidney tubules, was also noted exclusively in the male rats. Although current regulatory practice would consider humans to be potentially as sensitive to such effects as the male rat, it has been suggested that the male rat may be uniquely predisposed to renal toxicity from hydrocarbon exposure and may be an inappropriate animal model for human risk assessment. *In vivo-in vitro* DNA repair assays of unleaded gasoline have failed to produce evidence of genotoxic activity in isolated kidney cells of the male rat (37). These studies suggest that a direct genetic mechanism may not be operative. Knowledge of the actual mechanisms by which hydrocarbons induce toxicity and tumors in the male rat kidney will be essential to the interpretation of bioassay findings and their implications for human risk assessment.

Unleaded gasoline and other petroleum products contain five classes of hydrocarbons: normal paraffins, isoparaffins, cycloparaffins, olefins, and aromatics. The paraffins are saturated aliphatic hydrocarbons; the olefins and aromatics are unsaturated. Studies of the various component fractions of unleaded gasoline have indicated that the nephrotoxic activity resides primarily in those fractions that contain saturated branched aliphatic compounds (38). A series of 28-day gavage studies have revealed that the highly branched hydrocarbons such as 2,2,4-trimethylpentane and 2,2,5-trimethylhexane are the most active nephrotoxins in male rats (39). Studies of other hydrocarbon-containing compounds such as jet fuel, Stoddard Solvent, decalin, and methyl isobutyl ketone have also shown a similar pattern of nephrotoxicity in the male rat, but no notable effects in other species such as the dog or monkey (40).

The accumulation of proteinaceous material in the

form of hyaline droplets within the lysosomes of the proximal tubules of the male rat kidney was a common feature in these experiments. These findings suggest that renal protein handling is compromised by hydrocarbon exposure. The glomerulus is the functional unit of the kidney nephron that selectively filters low molecular weight proteins from the plasma. Most filtered proteins are not excreted in the urine but rather are reabsorbed by the proximal tubule cells where catabolic enzymes in the lysosome organelles degrade them into amino acids. However, untreated male rats exhibit proteinuria, with over 50% of the excreted protein consisting of α -2u-globulin. This low molecular weight, sex-dependent protein is synthesized in the liver under androgenic induction and then completely filtered from plasma at the glomerulus (41-43). Plasma levels of this protein in the female rat are very low, and it has not been detected in mice or humans. Although a large portion of the filtered α -2u-globulin is reabsorbed by the proximal tubules and recycled (44), its appearance in the urine of the male rat suggests that the reabsorptive capacity of the proximal tubule for this protein may be limited.

Alden et al. (40) have shown a clear correlation between toxicity and α -2u-globulin accumulation in the kidney using decalin as a model hydrocarbon. Studies with a specific antibody to this protein demonstrated that decalin-induced hyaline droplets in the male rat kidney were composed of α -2u-globulin. Kloss et al. (45), using another radiolabeled hydrocarbon, 2,2,4-[14 C]-trimethylpentane (TMP), showed 10-fold greater amounts of radiolabel in male rat kidneys than in female rat kidneys and no such accumulation in any organ of male mice. Additional experiments by these investigators have demonstrated that modulation of cytochrome P-450 metabolism alters the *in vivo* disposition of TMP, with inhibited metabolism leading to reduced renal retention of TMP. These findings suggest that production of a reactive metabolite of TMP in the liver may play an important role in the induction of nephrotoxicity via the following complex mechanism (46).

After oral exposure, TMP enters the male rat liver, where it is initially hydroxylated at C-4. Although this 4-hydroxyTMP metabolite could then be quickly attacked by conjugative enzymes that would deactivate the molecule and target it for urinary excretion, its highly hydrophobic and bulky tertiary butyl group may make the molecule sufficiently lipophilic to be retained within the endoplasmic reticulum long enough for a second hydroxylation (at C-5) to occur. This diol metabolite would be quickly oxidized to form a reactive α -hydroxyaldehyde metabolite of TMP, of which only a small amount is further oxidized and decarboxylated to produce CO_2 . The reactive aldehyde metabolite could then form Schiff base adducts with the α -2u-globulin protein as it is being synthesized in the liver. The altered protein would then be secreted by the liver and filtered at the glomerulus of the kidney, where the covalent association of this protein with the TMP metabolite may preclude its effective degradation by the lysosomal pep-

tidases. Continuous loading of the lysosomes with altered protein would lead to hyaline droplet formation, disruption of lysosome integrity, and intracellular release of catabolic peptidases, resulting in cell damage and death. These events would then stimulate a wave of restorative cell proliferation, which in turn could account for the hyperplastic and neoplastic kidney lesions induced by chronic exposure to unleaded gasoline and other hydrocarbons. Recent studies at CIIT have confirmed that TMP produces a marked increase in cell turnover in renal tubule epithelium of male rats (47).

At present, the details of this complex mechanism for hydrocarbon nephrotoxicity require additional validation in the laboratory. However, available information indicates the fundamental importance for understanding toxic mechanisms in interpretations of the discrepant bioassay data. If the nephrotoxicity of gasoline can be definitively linked to a toxicological event that is unique to the male rat, it can be concluded that similar events are not likely to occur in humans. Research on the mechanisms of action of hydrocarbons may also provide important insights into how other chemicals without demonstrable genotoxic activity produce tumors in animal test systems.

Formaldehyde

Exposure to formaldehyde has been associated with a variety of adverse health effects (48-50). It is known to cause eye, nose, and throat irritation, as well as dermal irritation and allergic contact dermatitis. Formaldehyde is a weak mutagen in some strains of bacteria, fungi, and *Drosophila* larvae. It is also a weak initiator and weak promoter of cell transformation in the C3H/10T1/2 cell culture system. Formaldehyde induces DNA-protein cross-links in cultures of bacterial and mammalian cells, and this phenomenon has now been detected in the nasal tissues of rats exposed to formaldehyde vapor via inhalation (51). An important finding reported by Kerns et al. (52) was that chronic inhalation exposure of Fischer 344 rats to 14.3 ppm formaldehyde gas for over 2 years induced a 50% incidence of nasal cavity squamous cell carcinomas, an otherwise rare tumor in rodents. In sharp contrast, only 1% of the mice identically exposed to 14.3 ppm and rats exposed to 5.6 ppm exhibited this tumor upon necropsy.

To properly assess the potential carcinogenic effects of formaldehyde exposure in humans, it is critically important that the already available extensive data base regarding formaldehyde toxicity be optimally used. A key issue concerns the form of the relationship between the doses administered during the course of a bioassay and the corresponding doses delivered to specific macromolecules in nasal target tissues. Regulatory agencies continue to rely on the assumption that administered and delivered formaldehyde doses are proportional, despite a considerable body of evidence that indicates that this is not the case.

Short-term inhalation studies have now established that the amount of formaldehyde that binds covalently

to the DNA of replicating cells in the respiratory epithelium of the rat nasal cavity is nonlinearly related to the airborne formaldehyde concentration (51,53). Following two 6-hr exposures to formaldehyde, significantly less binding occurred at airborne concentrations of 2 ppm or below than would be predicted by linear extrapolation from the binding observed at concentrations of 6 ppm or more. At 2 ppm, the ratio of the predicted level of binding (assuming linearity) to the observed level was approximately 3.5. At 0.3 ppm, this ratio was even larger (approximately 5.6). These ratios represent the factors by which the delivered dose in target tissue is overestimated when strict linear proportionality between the administered and delivered doses is assumed.

Related mechanistic studies have demonstrated that exposure of rats to formaldehyde via inhalation induces the respiratory depression reflex (54), inhibits mucociliary clearance (55,56), inhibits intracellular metabolism of formaldehyde (53), and stimulates cell proliferation (54,57), all as nonlinear functions of the airborne formaldehyde concentration. Inasmuch as each of these phenomena appears to be an important controlling factor in the relationship between administered and delivered formaldehyde doses (58), the nonlinear relationship between covalent binding to DNA in target tissues and airborne concentration is not unexpected.

The respiratory depression reflex mediates the inhaled dose, and mucociliary clearance mediates the fraction of the inhaled dose that penetrates the mucous layer covering underlying epithelial cells in the nasal cavity. Intracellular metabolism modulates the fraction of formaldehyde entering these cells that remain free to bind with cellular macromolecules including DNA. Important in this regard is the fact that formaldehyde is an essential biochemical that is normally present in all living cells. It is not surprising that efficient metabolic pathways exist for its detoxication, at least at low airborne concentrations. The rate of cell replication controls the fraction of DNA that is single stranded, and it is known that formaldehyde binds covalently only to single-stranded DNA (59,60).

The concept of delivered dose provides a convenient vehicle whereby these and similar biological observations can be incorporated directly into the risk assessment process in a meaningful and effective way. For example, use of the covalent binding observations as the measure of exposure in several commonly used dose-response models has been shown to result in low-dose cancer risk estimates that are considerably lower than corresponding estimates based exclusively on airborne concentration, irrespective of the dose-response model employed (61). At 1 ppm formaldehyde, the maximum likelihood estimate of risk obtained with the three-stage version of the multistage model was reduced over 50-fold.

However, it is important to recognize that the use of data obtained in short-term mechanistic studies for risk assessment rests on a number of assumptions which are as yet unverified. For example, it must be assumed at

present that such data are representative of conditions throughout the course of a long-term bioassay. The assumption that steady-state distribution and pharmacokinetics are achieved rapidly can only be tested with laboratory experiments that cover more extended periods of exposure. It must also be assumed that covalent binding to DNA or concentration of specific DNA adducts plays an important role in tumor induction. Hoel et al. (62) have argued convincingly that it is likely to be "biologically more meaningful to relate tumor response to concentrations of specific DNA adducts in the target tissue than it is to relate tumor response to the administered dose of a chemical." However, the assumption that such measures are directly relevant to tumor induction still remains to be validated in the laboratory.

It should also be emphasized that differences between species in any of the diverse factors that determine the quantitative relationship between delivered and administered doses can produce corresponding differences in the delivered dose and the attendant cancer risk, even when the different species are identically exposed to the same administered dose. For example, the remarkable disparity in tumor incidence among rats and mice identically exposed to 14.3 ppm in the formaldehyde bioassay can be explained by the corresponding difference between these species in the inhaled dose (54). Comparative studies with species that bear a closer resemblance to humans than rodents (i.e., subhuman primates) can assist in establishing the relevance of the data obtained with rodent models to the assessment of human risk from formaldehyde exposure.

Summary

The central theme that emerges from this review of some CIIT research is that interspecies differences in responses to chemical exposures should not simply be ignored in favor of ad hoc generalizations from the worst case. Such differences provide important clues to the underlying mechanisms by which chemicals exert their toxic effects. These differences must be carefully studied in depth and then explained in mechanistic terms before truly valid and scientifically defensible judgments regarding their relevance to human risk assessment are possible. In each case, many factors must be considered, ranging in scope from physiologic responses of the whole animal to specific chemical reactions with critical macromolecules in both target and nontarget organs. Identification of the actual mechanisms involved in chemically induced toxicity should eventually lead to the development of risk assessment models that more adequately reflect the unique biological and toxicological characteristics of different species-chemical combinations.

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