RESEARCH STRATEGY IN INDUSTRIAL TOXICOLOGY

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Abstract.....While much of industrial toxicology is observational in character, pursuit of specific research is needed to facilitate the overall evaluation of potential toxicity for man. Two such areas are the application of physiologic pharmacokinetic models to inter-species extrapolation of toxic effects and an understanding of the role of cellular oncogenes in the process of spontaneous tumor formation in animals. A physiologic pharmacokinetic model was developed for methylene chloride (MeCl_2) which describes the fate of MeCl_2 and its metabolic products in numerous species including the mouse, rat, hamster and man. This model has been used to predict specific tissue concentrations of critical metabolic reaction products in target tissues between animals and man. If it is assumed that toxicity is related to target tissue concentrations such methodology provides a means of relating interspecies toxicity to absorbed dose. This methodology precludes the necessity of using arbitrary factors in relating animal toxicity data to man.

A particular controversial issue in animal toxicology is the significance of the enhancement of animal tumors in tissues which already have a high spontaneous incidence. Without a better understanding of the basic process of spontaneous tumor formation it remains difficult to interpret results from chemical treatment. In particular spontaneous liver tumors in the B6C3F1 mouse have been shown to contain an activated cellular oncogene identified as H-RAS. The activated cellular oncogene is present in tumor tissue only and not in surrounding normal liver tissue. Of particular significance is the high frequency of activation in these mouse liver tumors (82%) compared to a 10-20% incidence of oncogenes present in a variety of human tumors. This suggests the ultra sensitivity of this mouse strain to liver tumor induction. Additional studies in progress are designed to determine whether genotoxic and nongenotoxic hepatocarcinogens show differences in oncogene activation.

INTRODUCTION

It is clear from the evaluation of a large number of studies that there are often considerable quantitative as well as qualitative differences between species when evaluating toxicity including carcinogenicity. Therefore evaluating potential risk for man from experimental animal studies requires perspective both on quantitative as well as qualitative differences. Toxicity and carcinogenicity observed in animal testing frequently is not a linear function of administered dose. One quantitative tool being developed is the incorporation of physiologic parameters into pharmacokinetic models to facilitate extrapolation of the predicted effect as a function of the dose and route of exposure. Regarding qualitative differences between species, a particular controversial issue is the relevance to man of the enhancement of tumors in animals at sites which already have a high spontaneous incidence. Examples of recent work illustrating the use of physiologically based pharmacokinetic modeling (PB-PK) with methylene chloride (Andersen *et al.*, 1986) and the detection of an activated cellular oncogene in spontaneous hepatocellular carcinomas in the mouse (Fox and Watanabe, 1985) are presented.

METHODS

Details of the methods for the physiologic pharmacokinetic model for methylene chloride and the oncogene study are given in Andersen *et al.* (1986) and Fox and Watanabe (1985) respectively.

RESULTS AND DISCUSSION

Physiologic pharmacokinetics of Methylene Chloride (MeCl₂)

Mathematical models are extremely useful in toxicology to help conceptually visualize and ultimately test hypotheses. In particular pharmacokinetic modeling has facilitated improvements in the interpretation of animal toxicity studies by providing a physiological basis for extrapolating from high to low doses, between species and routes of administration and characterizing nonlinear phenomena. In this technique, a computer model is constructed with compartments which correspond to actual tissues or tissue groups of the body. The size of these compartments, as well as the blood and air flows through these compartments, may be obtained from the medical literature. Once partition coefficients and metabolic constants have been determined, differential equations describing the dynamic mass balance in each compartment may be written. In the case of MeCl₂ two enzymatically mediated metabolic pathways, one involving mixed function oxidase (MFO) and the other involving glutathione transferase (GSH) must be considered. Figure 1 is the diagrammatic representation of the physiologically-based pharmacokinetic model for methylene chloride. The

objective of constructing this model was to be able to predict concentrations of relevant metabolites in specific target tissues and thus facilitate extrapolation of potential effects from high to low doses and from experimental animals to man.

Figure 1 Diagram of the physiologically-based pharmacokinetic (PB-PK) model utilized for methylene chloride. Tissues of the body are grouped into five compartments with similar flow and partition coefficients : Lung, Fat, Liver, Richly Perfused, and Slowly Perfused. Metabolism occurs in the Lung and Liver compartments. MeCl_2 enters the body through inhalation with absorption into pulmonary blood in the Gas Exchange Compartment, or by ingestion with absorption directly into the Liver compartment.

Model Validation

To test the reliability of the model, the model predictions were compared with several sets of blood concentration/time course data. One of the strengths of physiological modeling is the ability to use the same model to predict the disposition of materials in a target species from pharmacokinetic data gathered from another species. The model was used to predict blood levels of MeCl_2 in F344 rats inhaling 200 or 1000 ppm MeCl_2 for 4 hr (Andersen *et al.*, 1984). Blood levels of MeCl_2 were well described by the model both during the 4 hr exposure, and for periods up to 120 minutes following cessation of exposure (Figure 2a).

Experiments conducted with human volunteers (Schumann, 1984) were also evaluated. In these studies, healthy volunteers were exposed to either 100 or 350 ppm of MeCl_2 for 6 hr and samples of venous blood were collected during the exposure, and for periods up to 24 hr after cessation of exposure. This simulation may be regarded as the ultimate test of animal extrapolation, and the predicted values for humans were in excellent agreement with the experimental data (Figure 2b).

Figure 2 Validation of the PB-PK model with experimental data. Figure 2a presents data obtained in F344 rats during and following inhalation exposure to 1000 ppm (open symbols) or 200 ppm (closed symbols); and Figure 2b presents data obtained in humans during and following inhalation exposure to 350 ppm (open symbols) or 100 ppm (closed symbols). In each case, the simulated data is presented as a solid line, while the experimental data is shown with closed or open symbols.

Dose and Interspecies Extrapolation

The PB-PK model can now be used to calculate the values of the toxicologically relevant chemical species in various tissues under a variety of exposure conditions. To illustrate, Andersen *et al.* (1986) have shown that metabolites from the GSH pathway are likely relevant to the toxicity and oncogenicity observed at high doses in the liver and lungs of mice. Therefore assessment of risk in man necessitates estimation of the concentration of these metabolites in the target tissue of humans. The value of the liver metabolite concentration related to the GSH pathway in B6C3F1 mice and human following inhalation of various concentrations of MeCl_2 for 6 hr/day is plotted in Figure 3a.

The calculated metabolite concentrations are displayed on a log/log plot for exposure concentrations from 4000 ppm to 1 ppm. Metabolite concentrations for B6C3F1 mice are represented by the heavy solid line, while those for humans are represented by the heavy dashed line. Metabolite concentrations which would be obtained by linear extrapolation of data from the 4000 ppm mouse exposure are depicted as a lighter solid line in this and other figures. The concentration of metabolites calculated from the PB-PK model are close to those estimated by linear extrapolation with the mouse above 1000 ppm, but deviate from linearity in the region below 1000 ppm. The non-linearity in the curve is apparent in the region where the MFO pathway saturates. Saturation of MFO make a larger percentage of the MeCl_2 available for metabolism by the GSH pathway, resulting in a disproportionate increase in the metabolites of the GSH pathway at exposure concentrations above 100 ppm. Similarly in humans (exposed 6 hr/day) the curve also displays a non-linearity in the region between 100 and 1000 ppm although this is less pronounced than in mice. Importantly the values of the human metabolite concentrations are lower than the mouse throughout the entire exposure range.

Figure 3b presents the values of the lung dose of metabolites related to the GSH pathway in mice and humans exposed to various concentrations of MeCl_2 in air. In this case, the non-linearity in the mouse curve is of smaller magnitude than for mouse liver, indicating a smaller effect of MFO upon the material available for conjugation with GSH. As in the liver, the concentration of metabolites in human lung is lower than mouse at all concentrations probably because of the lower activity of GSH transferase in human lung relative to mouse lung.

As demonstrated, physiologic pharmacokinetics can be a powerful tool to facilitate extrapolation between doses and species. It can be used to generate and test hypotheses concerning mechanisms of action as well as facilitate extrapolation. Most importantly, if sufficient knowledge is available it allows direct interspecies comparisons for predicting potential effects which precludes the use of arbitrary factors which assume greater or lesser sensitivity of man compared to experimental animals.

Figure 3 Relationship of the metabolite concentrations associated with the GSH pathway to external dose. Figure 3a and Figure 3b show the values in B6C3F1 mice and humans during inhalation exposure (6 hr/day) in the liver and lung respectively. Data for mice are shown as a heavy solid line, while data for humans are shown as a heavy dashed line. In each case, a reference line depicting a linear extrapolation from the highest administered dose of MeCl_2 (4000 ppm) is shown as a light, solid line for comparison. Units for the Y-axis are mg metabolized per liter tissue per 24 hr.

Oncogenes and Animals Tests

The difficulty of interpreting bioassay data from animals which exhibit a high spontaneous background tumor incidence, and the relevance of an increased incidence associated from exposure with chemical agents to human risk assessment, is an area of particular controversy (Interdisciplinary Panel on Carcinogenicity, 1984 ; Task Force of Past Presidents-SOT, 1982 ; Nutrition Foundation, 1983). Resolution of this controversy necessitates a more detailed understanding of the cellular mechanisms of hepatic tumorigenesis in the B6C3F1 mouse.

Efforts to understand the molecular mechanisms of cancer have been aided enormously by the recent detection of cellular oncogenes (Shilo *et al.*, 1981). These genes seem to play a role in normal cellular proliferation and differentiation. However, aberrations resulting in qualitative changes in the structural gene or quantitative increases in the level of gene expression can lead to the transformation of a normal cell into a cancerous one (Reddy *et al.*, 1982 ; Schwab *et al.*, 1983).

In vitro DNA transfection studies using NIH 3T3 cells have detected cellular oncogenes in a variety of human tumors and tumor cell lines (Pulciani *et al.*, 1982a). While considerable effort has been expended in evaluating the role of oncogenes in human cancer, characterizing the presence or absence of cellular oncogenes in animals used for chronic carcinogenicity testing (i. e., B6C3F1 mouse) has only recently been undertaken (Fox and Watanabe, 1985 ; Reynolds *et al.*, 1986). Yet the vast majority of data on which decisions are made to protect the public from potential carcinogens are generated from animal bioassays. Understanding the potential role that cellular oncogenes play in tumor development of the B6C3F1 mouse is important for proper interpretation of the bioassay data from this strain and its proper use in assessing human risk. Experiments addressing this concept and discussion of the possible role that cellular oncogenes are anticipated to play in chemical carcinogenesis in the B6C3F1 mouse follow.

DNA from Spontaneous Liver Tumors Transforms NIH 3T3 Fibroblasts

The results from the transfection assay with DNA from spontaneously occurring liver tumor tissue derived from untreated 24 month old male B6C3F1 mice are shown in Table 1. Initial experiments demonstrated that the DNA isolated from 6 of 11 animals with spontaneous liver tumors was capable of inducing foci of transformed cells in the NIH 3T3 cell culture system (Table 1). The specific transforming frequencies of the tumor cell DNA ranged from .005-.02 focus forming units per microgram of DNA. These values are in close agreement to the frequencies reported by Reynolds *et al.* (1986) in their experiments with B6C3F1 mouse spontaneous liver tumor DNA, and for DNA from human hematopoietic tumors (Eva *et al.*, 1983). No foci were observed when NIH 3T3 cells were transfected with calf-thymus DNA or DNA isolated from the liver tissue of a non-tumor bearing 3 month old male B6C3F1 mouse.

To confirm the initial results, a second experiment was conducted using DNA isolated from the same 11 tumor bearing animals. Furthermore, the number of control cultures was expanded to include liver DNA isolated from two 3-5 month old and ten 24 month old male B6C3F1 mice without tumors. As was previously observed, foci were detected in the cultures transfected with DNA derived from the tumor tissue (Table 1). In this experiment 7 of the 11 tumor bearing animals were positive in the ability to induce transformed foci. Foci induction was not observed in either the 43 cultures transfected with DNA isolated from normal liver tissue of young mice (3-5 months old) or the 40 cultures transfected with DNA from normal liver tissue of 24 month old mice (Table 1). The lack of transforming activity of DNA derived from normal liver tissue of the 24 month old mice indicates that the transforming potential of the tumor DNA was not a unique function of the animal's age.

Table 1 Transfection of NIH 3T3 Cells with Spontaneous B6C3F1 Mouse Tumor DNA

DNA SOURCE	NO. FOCI OBSERVED	NO. ANIMALS SCORING POSITIVE TRANSFECTION
<u>EXPERIMENT # 1</u>		
^a 11 (24 MO.) B6C3F1 w/TUMORS	10/44 PLATES	6/11 (55%)
CONTROL		
CALF-THYMUS DNA	0/4 PLATES	
1 (3 mo) B6C3F1 w/o TUMOR	0/4 PLATES	0/1 (0%)
<u>EXPERIMENT # 2</u>		
^a 11 (24 MO.) B6C3F1 w/TUMORS	14/44 PLATES	7/11 (64%)
2 (3-5 mo) B6C3F1 w/o TUMORS	0/43 PLATES	0/2 (0%)
10 (24 MO.) B6C3F1 w/o TUMORS	0/40 PLATES	0/10 (0%)

^a Positive transfection was observed with DNA from 9 of 11 (82%) animals in at least one of the two transfection assays.

The combined results of these two experiments indicate that tumor DNA from 9 of the 11 animals was active in at least one of the two transfection assays. This represents an 82% positive transforming activity which is appreciably higher than that previously reported for DNA isolated from human tumor tissue (10-20%) (Eva *et al.*, 1983, Pulciani *et al.*, 1982b). This high incidence of transfection suggests that a common mechanism of oncogene(s) activation is occurring in tumor tissue from the B6C3F1 mouse.

Identification of the B6C3F1 Mouse Liver Oncogene

The transfection experiments described above strongly suggest the existence of an active cellular oncogene in the spontaneous liver tumor tissue of the B6C3F1 mouse. Positive confirmation required the identification of the active transforming gene within the tumor tissue. This was accomplished by isolating DNA from the primary NIH 3T3 cell transformants derived from the transfection experiments discussed above and subjecting it to Southern blot hybridization analysis using various oncogene probes. This analysis along with secondarily transfecting rat cells proved unequivocally that the transformation was due to the presence of the *H-ras* oncogene (Fox *et al.*, 1986).

Although the detection and identification of the active *H-ras* oncogene in the B6C3F1 mouse liver suggests that it is an important etiologic agent in the development of spontaneous liver tumors, the activation of *H-ras* alone is probably not sufficient to induce complete cellular transformation. In general, the number of oncogenes required to transform a normal cell into a fully neoplastic cell is an area of debate. Several investigators have demonstrated that at least 2 oncogenes are required to transform primary rodent cultures to a neoplastic phenotype (Land *et al.*, 1983 ; Ruley, 1983 ; Newbold *et al.*, 1983). These studies suggest that at least two genetic changes are required for cellular transformation. Therefore the involvement of cellular oncogenes in tumor development does not conflict but compliments the well established concept of multi-stage carcinogenesis.

One of the surprising outcomes of our studies was the high frequency of oncogene activation observed in the liver tumors. Of the tumor bearing animals examined, 82% contained the activated oncogene subsequently identified as *H-ras*. These results are strikingly similar to those of Reynolds *et al.* (1986) who reported that 77% (10/13) of spontaneous hepatocellular carcinomas from male B6C3F1 mice scored positive by DNA transfection (8 of 10 identified as *H-ras*). This is considerably higher than the 10-20% incidence detected in human tumors (Eva *et al.*, 1983 ; Pulciani *et al.*, 1982^b). This higher incidence indicates that the B6C3F1 mouse is dissimilar to the human population in the ability to activate, with high frequency, a transforming oncogene. This obviously has great impact in the extrapolation of carcinogenic risk between these two species and should be considered when making risk assessment decisions.

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