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Chemical Carcinogenesis

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Understanding the relationship of chemicals to carcinogenesis has progressed significantly since the initial observations of Hill and Pott in the 1700's. Distinguishing between DNA-reactive chemicals and those which increase cancer risk by increasing cell proliferation has been a major breakthrough in delineating overall mechanisms. Competing processes for activation versus inactivation of chemicals occur at many levels, including metabolism, DNA repair, and cellular repair processes. These processes can be affected by other agents to decrease carcinogenesis (chemoprevention). Increasing knowledge of the multiple steps of carcinogenesis is leading to improved methods for screening chemicals for carcinogenic activity and for regulatory decision making. Improvements in assessment of modes of action involved in animal and *in vitro* models have led to more rational approaches to assessing relevance to humans. The advent of genomics and high-throughput technologies have contributed to investigations of mechanisms and is beginning to impact development of better methods for screening chemicals. Based on developments in basic research, epidemiology, and astute clinical observations, the major risk factors and etiologic agents have been identified for a majority of cancers, which is beginning to lead to methods to decrease cancer incidence overall and identify targets for early detection and treatment.

Key Words: **Cell proliferation; DNA reactivity; non-DNA reactivity; carcinogenicity testing; human relevance.**

Chemical exposure has been related to the development of cancer ever since the observation by John Hill (Hill, 1761) that snuff users developed nasal cancer more frequently than the general population. However, chemical carcinogenesis generally dates specifically to the observation by Sir Percival Pott (1775) in 1775 describing the frequent occurrence of cancer of the scrotum in chimney sweeps in England. He hypothesized that this was because of their significant exposure to soot. More importantly, he also proposed a mechanism to reduce the incidence of these cancers simply by requiring these individuals to bathe on a regular basis. This was instituted, and the incidence of scrotal cancer was essentially eliminated. It is important to note, given subsequent scientific discoveries and emphasis on extrapolation to low exposure levels, that he did not recommend eliminating the exposure completely, merely reducing it. Scrotal cancer today is a rare disease.

Astute clinical observations such as that made by Pott (1775) have been the basis for the discovery of many of the currently known classes of chemical carcinogens in humans. Examples include the observation by Rehn (1895) in 1895 that workers in the aniline dye industry in Germany frequently developed bladder cancer and more recent observations concerning the induction of angiosarcomas in patients exposed to contrast material used for radiologic imaging studies (Vajdic *et al.*, 1986) and vinyl chloride exposure in the workplace in Louisville, Kentucky (Creech and Johnson, 1974).

Research based on these observations led to several seminal discoveries in the history of chemical carcinogenesis. Investigation of coal tar, e.g., led to the first experimental induction of tumors in animal models by Yamagiwa and Ichikawa (1915) by painting this material on the skin of rabbits and mice. These models have been used in carcinogenesis research ever since. Similarly, the relationship of soot to cancer induction led to the purification and identification of the first pure chemical carcinogen by Kennaway and Hieger (1930) when they purified a small amount of dibenz[a,h]anthracene and produced tumors by painting the chemical on the backs of mice. The observation by Rehn (1895) of bladder cancer in the aniline dye industry led to the discovery by Kinosita (1936) and Yoshida (1933) of the induction of liver cancer in rats by *o*-aminoazotoluene. This research also demonstrated the importance of dietary effects on the carcinogenic process.

Ultimately, 2-naphthylamine was identified as one of the principle chemicals to which workers in the aniline dye industry were actually exposed, and Hueper *et al.* (1938) demonstrated that it was a bladder carcinogen when administered to dogs. In their study, they were also the first to emphasize the importance of latency. Their experiment involved administration of 2-naphthylamine to dogs for 2 years. The long latency period

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related to the development of cancer after chemical exposure has been an important consideration in theoretical models of carcinogenesis and in epidemiology ever since and has been a major barrier to the identification of additional chemicals and their possible relationship to cancer. This was illustrated subsequently in numerous studies, such as demonstrating the relationship of cigarette smoking to lung cancer (and subsequently to other cancers), even though exposure frequently began during the teen years of the individuals, whereas lung cancers did not usually develop until after the age of 50 years. Even with high exposures to potent chemical carcinogens, such as 2-naphthylamine, benzidine, and vinyl chloride, the latency period is frequently 20–30 years or more. The basis for this long latency has posed a theoretical challenge to scientists continuing to today, but the necessity for multiple specific genetic errors occurring stochastically in a single cell provides the explanation for this latency (see below).

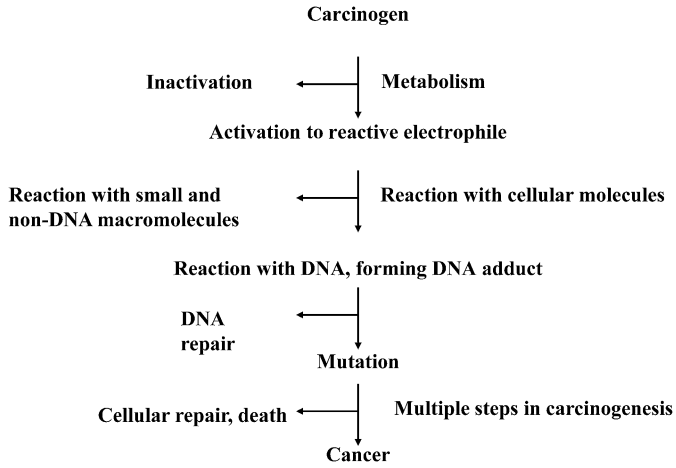

FIG. 1. Competing activating and inactivating processes for DNA-reactive carcinogens. In addition, these must occur in a pluripotential (tissue stem) cell, and the cell must replicate to fix the DNA alteration produced by a DNA adduct as a permanent mutation.

METABOLIC ACTIVATION VERSUS DEACTIVATION

Numerous chemicals have been identified with carcinogenic activity either in humans or in animal models. It was not until the publication by the Millers (DeBaun *et al*., 1970; Miller and Miller, 1977) in 1970 that a common mechanism could be demonstrated for many of the chemicals from a variety of chemical classes. They were able to demonstrate that an aromatic amide, 2-acetylaminofluorene (2-AAF), was metabolically activated to a reactive electrophile, which bound to DNA, forming DNA adducts and ultimately leading to mutations. Metabolic activation has been a mainstay of carcinogenesis research ever since. A variety of metabolic processes have been identified in the activation of a variety of classes of carcinogens, primarily involving cytochrome P450 enzymes, but numerous other enzyme systems have also been identified in the specific activation of chemicals (Guengerich, 2000).

It has also become apparent that nearly all chemicals undergo metabolism through several competing enzyme pathways, with differences in kinetics and saturation levels (Guengerich, 2000). A common group of enzymes involved with carcinogen metabolism are the cytochrome P450 isozymes. However, numerous other enzyme systems have been identified that participate in the metabolism of various carcinogens (Mitchell and Smith, 2010). Although some of these metabolic processes lead to activation to reactive electrophiles, many actually lead to inactivation of the chemicals by increasing aqueous solubility and leading to their increased excretion either in the urine or in the feces (Fig. 1). Thus, exposure to any chemical initiates competing metabolic pathways for activation versus inactivation. Detailed research over the past 20 years has shown that there are marked differences in the level of each of these enzyme processes in human individuals because of differences in activity of the enzymes secondary to single nucleotide polymorphisms (SNPs), as well as environmental influences on induction or inhibition of these enzyme systems (Boddy and Ratain, 1997). Although considerable research has been performed examining the role of these SNPs on susceptibility to carcinogenesis, this has not proven to be as influential as initially suspected. Frequently, the influence on cancer risk is less than twofold between the various individuals, even though the metabolic activities between specific enzymes can vary much more. This relatively small effect on cancer induction probably reflects the numerous alternative pathways as well as competing pathways for activation/inactivation of the chemical in addition to the multistep nature of the carcinogenic process.

One of the most extensively studied metabolic systems influencing activation and inactivation has been acetylation of aromatic amines (Hein, 2006). Individuals are generally classified as slow or fast acetylators, which is genetically determined. The specific isozymes and genetic variability have been extensively investigated. Aromatic amine acetylation dimorphism was actually the first of the differences identified, which led to the development of the field which now is commonly referred to as molecular epidemiology (Lower and Bryan, 1973). In humans, with respect to bladder cancer, acetylation of the aromatic amines is actually a deactivation process. Thus, one can hypothesize that individuals who are fast acetylators should have a decreased risk of developing bladder cancer in response to exposure to aromatic amines, in contrast to slow acetylators. Acetylation rates also influence the metabolism of a variety of drugs, such as the antituberculosis drug isoniazid, which can be clinically important. In several epidemiology studies investigating occupational exposure to chemicals and exposure to cigarette smoke, there is evidence that fast acetylators actually do have a decreased risk of developing bladder cancer compared with slow acetylators

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(Hein, 2006). However, it has been suggested that aromatic amines might also be related to the induction of colon cancer and even of breast cancer. In these instances, it appears that acetylation might actually be an activation step, so that fast acetylators would be expected to be at increased risk for developing these tumors (Shin *et al.*, 2008). However, for all these tumors, including the urinary bladder, the differences in susceptibility are relatively weak and have not been observed in all epidemiologic investigations examining this issue. Conflicting results have been reported for the examination of the influence of nearly all SNPs on the development of various cancers. However, this remains an active area of investigation and one involving a very complex interaction of variables. Not only is there the issue of competing metabolic systems for activation and inactivation of a given chemical but also there are alternative pathways, variability in induction, and inhibition of these enzymes because of environmental influences, as well as an interaction with numerous other chemicals that might be involved in the carcinogenic process for a given tissue in a given individual.

DNA Repair

The role of metabolic activation in carcinogenesis and the importance of DNA damage and mutation have led to additional avenues of research regarding mechanisms of carcinogenesis and influences on the carcinogenic process. The first of these is DNA repair (Hoeijmakers, 2009). Cells constantly deal with the formation of DNA adducts from either endogenous or exogenous influences (Greenfield *et al.*, 1984; Hoeijmakers, 2009; Phillips *et al.*, 2000), including DNA damage that occurs secondary to radiation. The fact that cells do not either automatically die or evolve into cancers because of these adducts is largely because of the fact that there is a remarkable system of DNA repair processes that eliminates these specific alterations in DNA so that mutation does not always occur. In fact, nearly all DNA adducts and damage are repaired, with only the rare exception leading to the change in sequence and mutation (Fig. 1).

The importance of DNA repair in the carcinogenic process was also demonstrated utilizing what Dr Robert Good (Good, 1968) referred to as experiments of nature, i.e., individuals born with specific mutations related to specific cellular processes. Cleaver (Cleaver *et al.*, 1975) identified that individuals with xeroderma pigmentosum (XP) had a markedly increased rate of development of skin cancers in response to sun exposure. He was able to demonstrate that the specific enzymes involved in the various forms of XP were related to repair of the DNA damage that was induced by ultraviolet (UV) radiation, the major causative factor of skin cancer in humans. Individuals with XP, depending on the specific site of mutation, had varying degrees of inability to repair this DNA damage. Thus, they had varying degrees of susceptibility to damage by the UV radiation and varying susceptibilities to developing skin cancer. Some of these individuals were exquisitely sensitive to the UV radiation and developed multiple skin cancers early in life unless they were completely protected from the UV radiation. For the development of cancer, ultimately DNA mutation must occur because of these alterations in the DNA sequence either because of single nucleotide changes or larger alterations in the chromosomes. Because these abnormalities each carry with them specific repair processes, polymorphisms in the enzymes involved can lead to differences in susceptibility, just as differences in susceptibility can be because of variations in metabolic activation and inactivation processes.

Genotoxicity Assays

The second major consequence of the observation that carcinogens were metabolically activated to bind to DNA leading to mutation was the development of a variety of genotoxicity assays, beginning with the Ames assay in various strains of *Salmonella* bacteria (Gee *et al.*, 1994; McCann *et al.*, 1975). Ames *et al.* (1973) developed strains of *Salmonella* that were particularly susceptible to DNA damage, but more importantly, they provided an exogenous source of metabolic activation systems by adding induced liver microsomes to the medium along with the test chemical and the test strain of bacteria. This addressed the issue of metabolic activation for many of the chemicals being analyzed because the enzymes involved in such processes frequently are highest in the liver. This took advantage of the requirement for metabolic activation for most DNA-reactive carcinogens. Utilizing the Ames assay, numerous investigators were able to show a strong correlation between positivity in this assay with a positive response in rodent bioassays for carcinogenesis. This led to the now infamous statement that carcinogens are mutagens and mutagens are carcinogens.

A plethora of genotoxicity assays have been developed during the past four decades, which were designed not only as a means of identifying a mechanism by which a chemical can produce cancer but also as a short-term screening test for identifying chemical carcinogens (Kirkland *et al.*, 2007). Although not a major focus of this discussion, besides the specific mutation assays such as the Ames assay, many of the other assays have proven extremely difficult to interpret, especially regarding their relevance for predicting carcinogenicity (Kirkland *et al.*, 2007). Many have a predictive value for carcinogenesis less than 50%, less than a coin flip. This is because many of these assays are *in vitro* and involve considerable levels of cytotoxicity. They also involve indirect effects on DNA and chromosomes rather than a direct interaction with DNA. Such indirect assays include assessments for sister chromatid exchange, chromosomal aberrations, and micronuclei. Furthermore, many of these *in vitro* assays cannot be verified *in vivo*. Several *in vivo* genotoxicity assays have also been developed, including specific point mutation assays involving specially designed mice and rats, such as the

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Big Blue rat, Big Blue mouse, and Mutamouse (Lambert *et al.*, 2005), as well as *in vivo* micronucleus assays, the Comet assay, and others. However, a more restrictive definition of genotoxicity is to limit the effects to DNA reactivity specifically. This would include chemicals that react directly with DNA or following metabolic activation to form DNA adducts (Phillips *et al.*, 2000). Only those adducts that are mutagenic (not all adducts have mutagenic potential) will lead to mutation and have the potential to increase the incidence of cancer.

Extensive computer analyses relating three-dimensional chemical structure to DNA reactivity have resulted in a refined structure-activity relationship (SAR) process (Tong *et al.*, 2003). Several computerized models are now available for this analysis, which can relate not only correlation between chemical structure and metabolic activation to DNA reactivity but also for a variety of other biological targets, such as specific cell receptors. However, for a correlation to carcinogenicity, the only significant correlation appears to be with identifying a chemical that can be directly or metabolically activated for DNA reactivity. For chemicals that induce cancer by mechanisms that do not involve DNA reactivity, these models are of little value. However, such SAR models are of considerable value in screening new chemicals for commercial development, with the ability to exclude those that have the potential for DNA reactivity. Ultimately, the ability of a chemical to be DNA reactive can be demonstrated by an evaluation of DNA adduct formation either *in vitro* or, better, *in vivo*. Especially now that there are extraordinarily sensitive techniques available for assessing DNA adducts, this is considered the best and most definitive method for demonstrating DNA reactivity. Demonstrating DNA adduct formation *in vivo* in the target tissue is best because *in vitro* findings do not always translate to *in vivo* effects. Furthermore, possible reactive chemical substituents identified by SAR do not always relate to actual metabolic activation and DNA adduct formation *in vivo*. For example, although numerous aromatic amines can be metabolically activated to DNA-reactive substances, there are numerous aromatic amines that are not metabolically activated and do not form DNA adducts. These include not only substances to which we are exposed exogenously but also substances that are generated endogenously through normal intermediary metabolism, such as kynurenine, anthranilic acid, and other metabolites (Bryan, 1969; Seifried *et al.*, 2006). 2-Naphthylamine is metabolically activated to a reactive electrophile and is mutagenic and carcinogenic. Its isomer, 1-naphthylamine, is not metabolically activated and is neither mutagenic nor carcinogenic (Clayson and Cooper, 1970).

DNA Reactive versus Non-DNA Reactive

Although these genotoxicity assays, specifically DNA reactivity assays, have been a major development in chemical carcinogenesis, both with respect to determination of mechanism and for screening new chemicals for potential genotoxic activity, it was already apparent at the time when many of these assays were being developed in the 1970's that there were a large number of chemicals that produced cancer in animal models (as well as in humans) that were not positive in any of these assays. These included such chemicals as phenobarbital (Whysner *et al.*, 1996), saccharin (IARC, 1999), as well as others. Weisburger and Williams (1981) were the first to publish an article stating that there were essentially two classes of chemical carcinogens, genotoxic and nongenotoxic. They referred to the nongenotoxic carcinogens as epigenetic. Refinement of this dichotomy is to separate chemicals on the basis of DNA reactivity. As discussed below, those chemicals increasing the risk of cancer that are not DNA reactive do so by increasing the number of DNA replications in the target cell population (increased cell proliferation). Distinction between classes of chemicals based on their ability to generate DNA reactivity is the basis for the classification of chemical carcinogens to this day and forms the basis for the distinction of potential risks to humans in regulatory decision making.

Multistage Carcinogenesis

A major focus of carcinogenesis research has been the issue of latency. A major basis for this delay in the development of cancer secondary to exposure to chemicals or other carcinogenic agents is that cancer is a multistep process. The concept that cancer involves multiple steps was already being investigated as early as the 1930's, but it was not until the seminal publication of Berenblum and Shubik (1947) in 1947 that a model distinguishing steps in carcinogenesis was actually demonstrated. This model was referred to as initiation and promotion and was later amended to include a third step, progression. In their model, Berenblum and Shubik (1947) showed that promotion actually involved clonal expansion of initiated cells to form benign lesions, with progression being the step needed to convert these benign lesions to malignancy.

The two steps of the carcinogenic process, initiation and promotion, were shown to have distinct qualities (Berenblum and Shubik, 1947; Boutwell, 1964; Dragan *et al.*, 1993). To begin with, it was shown that the sequence of administration was critical, so that the initiator had to be administered first followed by the promoter. Also, it was subsequently shown that initiation was an irreversible event, what we now realize to be a permanent alteration in the DNA, whereas promotion could be reversible, at least until the step of progression to a malignancy occurred. Even the benign tumors that were produced in the Berenblum and Shubik (1947) model, skin papillomas on the backs of mice, could revert to normal. Another characteristic of initiation was that the effect appeared to be additive. If a dose of the initiator was fractionated into multiple parts but the cumulative amount administered remained the same, similar tumor incidences and numbers

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were induced. In contrast, the dose of the promoter was found not to be additive. There appeared to be a specific minimum fractional exposure that was required for promotion to occur, i.e., a threshold. These basic concepts have evolved into current perspectives on mechanism as well as regulatory decision making regarding chemicals.

In reality, we now know that initiation (and progression) is essentially the process of producing irreversible DNA damage, and initiators are chemicals that are DNA reactive, either directly or following metabolic activation (Dragan *et al*., 1993). Promoters, in contrast, are non–DNA-reactive chemicals and, as discussed below, produce their effect by increasing cell proliferation. Nearly all DNA-reactive carcinogens produce toxicity when administered at high doses, which will lead to increased cell proliferation, a promoting effect. This has given rise to the term complete carcinogen. As described in detail below, the terms initiation, promotion, and complete carcinogen become difficult to define except to classify chemicals as having the properties of DNA reactivity, increased cell proliferation, or both. Also, DNA replication by itself can lead to a "spontaneous" alteration in the DNA, effectively acting to produce initiation.

Although the model of initiation and promotion has served the carcinogenesis community well with respect to investigating the multistep nature of carcinogenesis and the carcinogenic properties of chemicals, there actually are several difficulties with this model (Cohen, 1998a, 1998b; Cohen and Ellwein, 1991). To begin with, administration of the promoter prior to the initiator has been shown to actually increase the carcinogenic effect. Furthermore, initiation-promotion assays have traditionally been performed in short-term experiments, usually 20 weeks or less in mouse skin studies, whereas evaluation of these chemicals, initiators and promoters, in a full 2-year bioassay, today's standard, showed they were carcinogenic in their own right. Furthermore, this model requires the presence of an intermediate clonal expansion of initiated cells for the development of the ultimate malignancy. Although in many animal models this occurs, such as mouse skin, rat liver, mouse and rat colon, mouse and rat lung, and mouse and rat urinary bladder, this is not always the case either in rodents or especially in humans. There are numerous malignancies in humans that occur without an intermediate benign lesion identifiable, such as certain leukemias, most sarcomas, small cell carcinoma of the lung and other tissues, and others. The malignant potential in these tumors appears to occur with the initial transformation of the cell without an intervening benign proliferation. As described below, a more generalizable multistage model can incorporate the concepts of the initiation-promotion model but also includes other factors.

A second multistage model was proposed by Armitage and Doll (1954) based on epidemiologic observations. They recognized that the incidence of many cancers increased exponentially with age. They derived an equation to address this observation and noted that by addressing the specific data

Armitage-Doll Equation for Multistage Carcinogenesis

$$I(t) = N \lambda_o \lambda_1 \ldots \lambda_{n-1} t^{n-1}/(n-1)!$$

FIG. 2. Formula derived by Armitage and Doll (1954) to describe the exponential increase in incidences of tumors with age. $I(t)$ = incidence at time t; N = number of normal stem cells; λ = rate of transition between stages; n = number of stages. N and λ are assumed to be constant, and cell proliferation rates are also assumed to be constant.

for a given tumor that the number of stages involved in the carcinogenic process could be identified (Fig. 2). This formula fitted the data well for cancers of many organs, such as lung and urinary bladder. However, there are many examples for which this model does not fit (Fig. 3). For example, in breast cancer, there is a bimodal appearance in the curve for times of increasing incidence, the perimenopausal time period and later in age (Moolgavkar and Knudson, 1981). Another example is Hodgkin's disease in the United States (Watanabe *et al*., 1986). Again, there is a bimodal distribution of increased incidences, one in the twenties and thirties and then again in later age. An even more striking example is testicular germ cell tumors in males, where there is a dramatic increase in incidence between ages 20 and 40, but these tumors rarely occur after the age of 50 (Kodama and Kodama, 1998). And, of course, there are the tumors that occur only or predominantly in childhood, such as retinoblastoma (Knudson, 1971).

In the approach developed by Armitage and Doll (1954), implicit assumptions are made that frequently are valid but in specific instances are not. One of these assumptions is that the

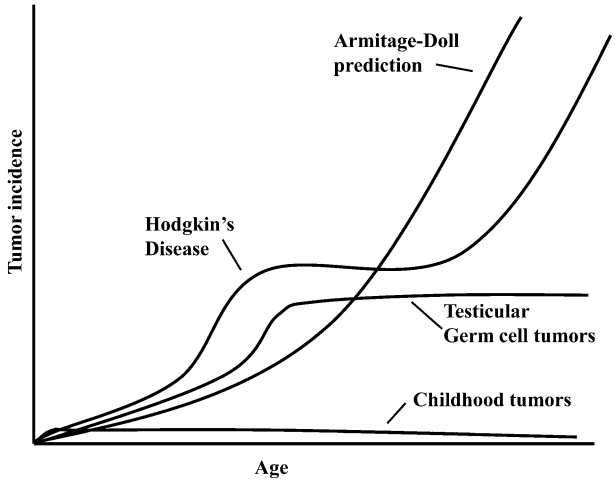

FIG. 3. Incidence curves for various types of tumors relative to age. The Armitage-Doll model predicts an exponential increase of tumor incidence with age, such as seen for cancers of the colon, prostate, urinary bladder, lung, and others. However, Hodgkin's disease has one major increase during the second to fourth decades of life in the United States, with another increase later in life. Testicular germ cell tumors have peak incidences during adulthood from the third to fifth decades, being rare after age 50. Childhood tumors, such as retinoblastoma, occur virtually exclusively in childhood. The curves are drawn to represent variations in shape and are not drawn to scale with respect to each other.

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rate of cell proliferation in a given tissue remains constant throughout lifetime, and another is that the number of target cells remains constant throughout the lifetime. Both of these variables can be influenced by normal growth and development and by exogenous factors. For example, osteosarcomas have peak incidences during the major growing period of humans, ages 15–25. This of course is when the bones are growing and osteoblasts are proliferating at a much greater rate and number than in the adult.

A third multistage model was proposed by Knudson (1971) in a landmark 1971 publication describing the occurrence of retinoblastomas in children. Until Knudson's publication, retinoblastoma was frequently considered an autosomal dominant disorder because nearly all children who inherited the disorder developed tumors. However, he posed the question as to why individuals who had inherited this disorder did not have all their retinoblasts develop into tumors. Although individuals who had inherited retinoblastomas usually had bilateral disease and multiple tumors in each eye, it was still a limited number, with most of the retinoblasts in the children's eyes being morphologically normal, never becoming malignant. In contrast, individuals who had the sporadic disease usually had unilateral disease and usually only had one tumor.

Knudson (1971) postulated that the retinoblastoma gene acted as an autosomal recessive gene and that individuals who had the inherited genetic abnormality actually had damage to one of the alleles in all their retinoblasts, whereas the other retinoblastoma allele that was inherited was normal. During normal growth and development, retinoblasts proliferate. In an occasional cell, the second allele spontaneously developed an abnormality, leading to the development of the malignancy. In contrast, in individuals developing retinoblastomas on a sporadic basis, a spontaneous error had to occur in both alleles during normal growth and development during cell replication of the retinoblast. Even though spontaneous errors in DNA are rare events during replication, the number of replications occurring normally during development provides sufficient opportunity for an allele to become abnormal. In an individual with one allele already abnormal, the child virtually always will develop an abnormality in the second allele at least once and usually in multiple such cells, leading to the development of multiple tumors. However, having two rare events occur in the same cell during normal development would be an extraordinarily rare event; approximately one individual per million births would be expected to develop such an abnormality. This is roughly the incidence of retinoblastoma in the general population on a worldwide basis. The necessity for cell replication becomes obvious once the eye fully develops and the retinoblasts stop replicating; tumor induction no longer occurs! Although for many cancers more than two events are required, in contrast to retinoblastoma, nevertheless, the general principles identified by Knudson in his model readily address the inadequacies of both the initiation-promotion model and the Armitage-Doll model.

It is obvious that the retinoblastoma gene as depicted by Knudson foresaw the definition of a tumor suppressor gene. That is, under normal circumstances, when the gene is functional, it actually inhibits the formation of cancer. It is only with the development of an abnormality in both alleles that this inhibition on cancer development is released and cancer occurs.

In contrast to tumor suppressor genes are the oncogenes (Hahn *et al*., 1999; Knudson, 1993). These are a group of genes that appear to act in a dominant fashion and when activated, either by a mutation or by other cellular processes, increase cell proliferation and produce cancers. In general, all tumor suppressor and oncogenes that have been identified are related in one way or another to cell proliferation or to the preservation of the DNA sequence, either directly or indirectly. A sequence of oncogene activation and tumor suppressor gene inactivation has been best defined for colonic adenocarcinoma by Vogelstein and others (Wood *et al*., 1996). These models provide a molecular basis for the Knudson hypothesis more generally and for the model described below. However, in the colon model and in others, it remains difficult to define exactly which genes are essential to the carcinogenic process and which ones modify the cell number and/or replication rate and/or mutation frequency.

An incredibly astute series of observations published by Boveri (1914) in 1914 actually described the fundamental concepts of oncogenes, genes activating cancer, as well as tumor suppressor genes, genes inactivating cancer development. Keep in mind that this was at a time when chromosomes were first being identified as genetic material and DNA had not yet been identified as the source of heredity, nor had modifications in DNA structure or sequence by exogenous or endogenous factors been discovered. These seminal, astute observations were essentially ignored by the scientific community for several decades.

In the early 1980's, a more generalized multistage model of carcinogenesis was developed by Moolgavkar and Knudson (1981) utilizing epidemiology data and by the Cohen laboratory (Greenfield *et al*., 1984). These models are based on a two-step process but can easily be broadened to a multistep process, as it has become apparent that for most cancers more than two steps are involved (Fig. 4).

Cell Proliferation and DNA Reactivity in Carcinogenesis

The target cell population in a given tissue is assumed to be the pluripotential cells in that tissue (Cohen and Ellwein, 1990a; Greenfield *et al*., 1984; Moolgavkar and Knudson, 1981). Cells that have already committed to differentiation or are fully differentiated are not the targets for carcinogenesis because they are ultimately destined to die. It is only the pluripotential cells (also referred to as tissue stem cells) that have the potential to develop as malignancies. In contrast, many benign tumors actually arise from cells that are already committed to differentiation.

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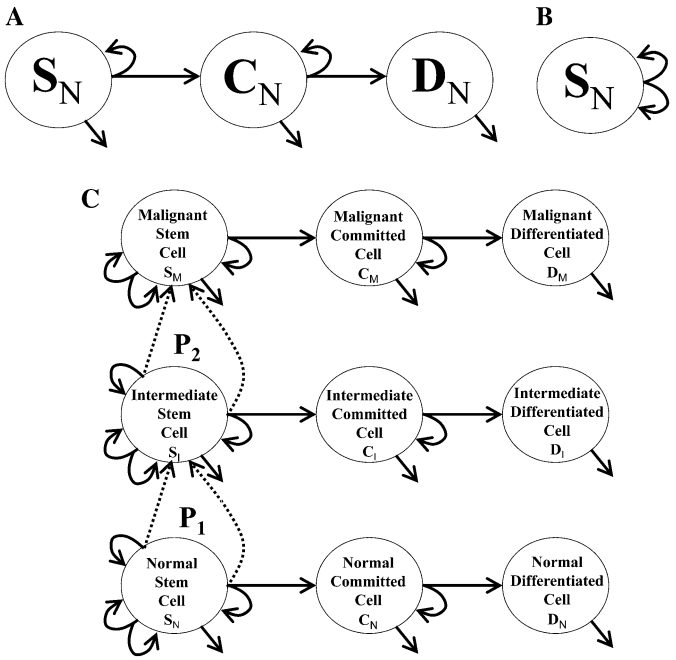

FIG. 4. Cellular processes of differentiation and carcinogenesis. (A) Tissues under normal conditions are composed of pluripotential stem cells (S_N), which divide into a duplicate stem cell and a cell committed to differentiation (C_N). Committed cells may undergo cell replication and/or undergo terminal differentiation (D_N), depending on the specific tissue. Terminally differentiated cells are destined to die (downward arrow) and be replaced. Stem cells and committed cells can also die (downward arrows). (B) Under special circumstances, such as direct mitogenesis or cell death with replacement, the stem cells divide into two stem cells rather than one stem cell and one committed cell. (C) Every time a normal stem cell (S_N) replicates, a mistake can occur in a critical part of a gene essential for converting the cell ultimately into a malignant cell. The rate of a mistake occurring is indicated as the probability p_1. This converts the normal stem cell into a stem cell in an intermediate population between normal and malignancy. The intermediate stem cell (S_I) can undergo the same replication and differentiation processes as the normal stem cells, forming committed (C_I) and differentiated (D_I) cells in the intermediate cell population. As the intermediate stem cells replicate, they also can produce errors in the DNA, at probability p_2, leading to the next step in the carcinogenic process. If two steps are required, this second error produces a malignant stem cell (S_M), which also can duplicate itself or undergo commitment (C_M) to differentiation (D_M). If more than two steps are required, there will be additional intermediate cell populations. Chemicals can increase the risk of carcinogenesis by affecting the probability of mistakes with each DNA replication (increasing p_1 or p_2), which represents a DNA-reactive process (DNA-reactive carcinogens), or it can increase the number of DNA replications in the normal and/or intermediate stem cell populations. Because the critical parameter is number of DNA replications, this can be increased by increasing the number of cells in these stem cell populations and/or increasing their rate of replication. (Part C is modified from Greenfield *et al.*, 1984, with permission.)

Under normal circumstances when a pluripotential cell divides, it produces another cell identical to itself and another cell that is committed to differentiation and ultimately undergoes terminal differentiation (Fig. 4A). Committed cells and terminally differentiated cells are destined to die. Thus, inducing mistakes in the DNA of these cells will not produce a tumor that can evolve with all the characteristics of a malignancy. Stem cells also can die, commonly killed by a variety of processes including necrosis and apoptosis. If these cells die, the remaining daughter cells can divide to form two daughter stem cells to replace the lost cells, or stem cells can be directly stimulated to replicate into two daughter stem cells (direct mitogenesis) (Fig. 4B). If there is injury, this is the way tissues are repaired and how this population can be replenished. Direct mitogenesis will lead to an increase in the number of cells that are present as well as usually inducing replication at an increased rate.

During DNA replication in the pluripotential cells, whether under normal circumstances generating one daughter cell and a committed cell or whether duplicating itself into two pluripotential cells, mistakes can occur in the DNA, which are fixed permanently and inherited by the cells (Fig. 4C). If the mistake occurs in the portion of a gene that is critical to the development of a given cancer for that tissue type, then the cell has taken a step toward the development of malignancy and enters into what we refer to as the intermediate cell population. This population can be clonally expanded, as described in the initiation-promotion model, but this does not have to occur.

Cells in the intermediate population can undergo the same processes as normal cells, i.e., they can replicate yielding one of themselves and one committed cell with terminal differentiation or they can replicate yielding two identical daughter cells. Again, during DNA replication of these intermediate pluripotential cells, mistakes can occur in the DNA. If the mistake is in the critical portion of a gene that is critical in the pathway to developing cancer in that tissue type, then that step will progress further toward malignancy. If it is a two-step process, then the second abnormality that occurs in this intermediate cell population will lead to a malignancy. If there are more than two steps, there will be multiple intermediate cell populations that are required for the ultimate development of malignancy.

Once the final alteration occurs and malignancy develops, in contrast to what has long been held as true for malignancies, not all these malignant cells will replicate and some of them can yield fully differentiated cells. This is the basis for tumors ranging from well differentiated to poorly differentiated. In well-differentiated tumors, the sequence of pluripotential cell to committed cells to fully differentiated cells is maintained, albeit at a slower rate than under normal circumstances so that a greater proportion of the cells in the tissue remain as the pluripotential cells rather than the fully differentiated cells. In contrast, poorly differentiated malignancies have few cells that progress in this direction, and most remain in the pluripotential stem cell population. Even in these cells, the proliferation rate is not uniform, and only a relatively small proportion of the cells will replicate in any given day. There is only one tumor that we are aware of that actually yields replication of nearly all the pluripotential cells on a daily basis and that is Burkitt's lymphoma (Cooper *et al.*, 1966).

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Fundamental to this model is that the transitions from normal to intermediate to malignancy occur during DNA replication, each with a certain probability that is usually very low. It is a stochastic process. The stochastic nature of carcinogenesis combined with the multiple DNA abnormalities that are required form the basis for the latency period required between chemical exposure and cancer development.

Based on this sequence of events, there are fundamentally only two mechanisms by which a chemical or any other agent can increase the risk of cancer. The chemical can increase the probability that at each replication of the pluripotential stem cell, there will be a mistake in a critical gene, the number of replications in this population can increase, or a combination of these two events (Cohen and Ellwein, 1990a; Greenfield *et al.*, 1984; Moolgavkar and Knudson, 1981). Chemicals that increase the probability of a genetic abnormality with each DNA replication are essentially those that are DNA reactive. Those that increase the amount of DNA proliferation as the basis for increasing cancer risk are those classified as non-DNA reactive. For nearly all DNA-reactive carcinogens, if the dose is high enough, there is also an increase in self-proliferation, usually because of regeneration following cytotoxicity.

Increased cell proliferation can occur either by an increase in cell births or a decrease in cell deaths (Cohen, 1998b). A decrease in cell deaths will lead to an accumulation of cells. Even if they are replicating at the normal rate, this still represents an increase in the number of cells that are replicating. This is a common mistake made in interpreting data on cell proliferation because many investigators focus on cell rate rather than on the actual number of replications. For example, with chemicals such as phenobarbital, there is a transient increase in DNA replication rate in the liver, but this is accompanied by a growth in the liver with an increase in the number of target hepatocytes (Whysner *et al.*, 1996). Thus, for the remainder of the animal's time exposed to phenobarbital, there actually is an increase in the number of cells replicating, but not the rate. Furthermore, it is critical that this proliferation occurs in the stem cell population. An increase in proliferation in those cells that are committed to differentiation or even fully differentiated will not lead to an increase in cancer risk. This is another common difficulty in interpreting cell proliferation data.

Synergy between Cell Proliferation and DNA Reactivity

A landmark study in carcinogenicity research is the ED01 mega-mouse experiment performed in the 1970's at the National Center for Toxicological Research. In this study, 2-AAF was administered to female mice for up to 33 months, with group sizes sufficiently large to detect an increased incidence of tumors of 1%, rather than the usual 2-year bioassay which has a detection limit of approximately 10% (Cohen and Ellwein, 1990b; SOT, 1981). The doses used were substantially lower than those typically used in 2-AAF experiments, ranging from a low dose of 30 ppm to a high dose of 150 ppm. At these doses, there was approximately a linear dose-response relationship for liver tumors, but for urinary bladder tumors, there was an increased incidence of tumors only at doses of 60 ppm and above. However, studies of DNA adduct formation showed steady-state levels in both tissues that were linear with dose, extending to doses much lower than those used in the carcinogenicity bioassay (Poirier *et al.*, 1989). In the liver, the tumor effect was due only to the interaction of 2-AAF with the DNA of the normal hepatocytes, with this interaction occurring much less frequently in the intermediate cell population (foci cells) (Cohen and Ellwein, 1990b). There was no apparent increase in cell proliferation in either population compared with untreated controls. In contrast, at low doses of 2-AAF, the effect of DNA reactivity on the bladder was insufficient to produce a detectable incidence of tumors. Keep in mind that the normal mouse bladder only has approximately 25,000 pluripotential cells, and therefore, only by increasing this target cell population and their rate of replication, which occurred at the higher doses, was a detectable incidence of tumors observed.

This synergistic interaction between DNA reactivity and cell proliferation has been identified in several model systems and in humans. For example, N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide (FANFT) is a bladder carcinogen in rats similar to 2-AAF, with a detectable incidence in the 2-year bioassay occurring only at doses that also increase cell proliferation (hyperplasia) (Murasaki and Cohen, 1983). At lower doses, the DNA reactivity is still present, but the effect does not produce a detectable incidence of tumors. In contrast, sodium saccharin at very high doses is not DNA reactive but increases cell proliferation in the urinary bladder epithelium. In a standard 2-year bioassay, it increases tumor incidences by approximately 1%. When FANFT administered at a dose that produces DNA reactivity but does not increase cell proliferation or a detectable incidence of tumors is coadministered with a dose of sodium saccharin that increases cell proliferation, a detectable incidence of tumors is identified.

In humans, examples also have been identified. For example, the aromatic amine 4-aminobiphenyl (4-ABP) is present in cigarette smoke and is excreted in the urine in forms that lead to DNA adduct formation (Cohen *et al.*, 2006). However, the amounts present in cigarette smoke are at levels that would be expected to be insufficient to induce the incidence of bladder tumors in humans that is actually observed. In addition to the DNA adduct formation, however, there is also an increase in proliferation of the urothelium in cigarette smokers. The cause of this increased proliferation has not been identified but would not be expected to be because of the 4-ABP. However, this combination of increased mutagenic DNA adduct formation by 4-ABP and increased cell proliferation leads to a significant incidence of bladder tumors, with cigarette smoking being the major cause of bladder cancer in the United States.

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The interaction of multiple agents has also been identified in liver carcinogenesis involving aflatoxin and hepatitis B virus (HBV) (Chuang *et al*., 2009). In parts of China, there is exposure to high levels of aflatoxin, a DNA-reactive carcinogen, but without HBV exposure. The estimated increase in risk of liver cancer is approximately two- to threefold compared with individuals not exposed to high levels of aflatoxin. In other parts of China, HBV is prevalent but without high levels of aflatoxin exposure. In these circumstances, the estimated increased risk is approximately 10–12 times. However, in populations where exposure to HBV is combined with high levels of aflatoxin, an exposure that combines increased cell proliferation with DNA reactivity, there is approximately a 65-fold increase in liver cancer risk, again illustrating the synergistic response that occurs when DNA reactivity is combined with an increase in cell proliferation.

Chemoprevention

To increase cancer risk, a chemical either increases DNA reactivity leading to an increase of mutation with each DNA replication or increases the number of DNA replications in the target cell population. To reduce cancer, the reverse of this must occur. Either the amount of DNA reactivity must be decreased and/or the amount of DNA replication needs to be reduced (Cohen, 1991). This has been the basis for chemoprevention since the initial postulate by Wattenberg (1985) created the concept of chemoprevention. This concept is based on the idea that consumption of exogenous substances could actually prevent certain types of cancer, especially those caused by chemicals. Research on chemoprevention in animal models and in human clinical trials has continued extensively ever since. However, in all these systems, the ultimate effects are to reduce the formation of DNA adducts by interfering with the metabolic activation, increasing metabolic deactivation, or binding to the reactive electrophile once it is formed so that it cannot react with the DNA or the effect reduces cell proliferation in the target tissue. However, like carcinogenesis itself, the issue is much more complex than originally imagined. Many of the examples of chemoprevention require doses that are in excess of what can be safely consumed by humans. Furthermore, in some examples where substances were used for chemoprevention, side effects of the agent actually led to an increase in cancer risk, as was seen in the beta-carotene trials in cigarette smokers (Hennekens *et al*., 1996), or led to an increase in side effects with other disease processes, such as the cyclooxygenase-2 inhibitors which had chemopreventive activity in animal models, but in humans have led to an increased risk of significant cardiovascular events (Marnett, 2009; Wu *et al*., 2010).

There is epidemiologic evidence that certain exposures can reduce cancer risk, such as increased consumption of fruits and vegetables, although this has been challenged more recently (Vastag, 2009). Caloric restriction was identified as a preventive process in animal models nearly seven decades ago (Kritchevsky, 1999; Tannenbaum, 1940) and has been demonstrated to be related to a reduced risk of certain cancers in humans (Lutz and Schlatter, 1992). In the tissues that are affected, caloric restriction not only reduces cell proliferation (Lu *et al*., 1993) but also has numerous other effects, such as effects on DNA repair, that could contribute to the inhibitory process (Hart *et al*., 1995). Although chemoprevention in the clinical setting has thus far been disappointing, this area of research continues.

Immunosuppression and Carcinogenesis

In the late 1950's and early 1960's, several investigators made observations that suggested that neoplastic cells developed foreign, specific antigens as they formed and that malignancy developed as a consequence of suppression of the normal immunosurveillance of foreign antigens, allowing the malignant cell to escape surveillance and develop into a cancer (Baldwin, 1973; Burnet, 1957, 1964; Klein and Klein, 1986; Kripke and Borsos, 1974; Schwartz, 1975; Thomas, 1959). Specific antigens were identified in mouse tumors that were related to certain malignancies and were immunogenic upon transplantation (Klein and Klein, 1988; Kripke and Borsos, 1974). Furthermore, many of the chemicals identified as carcinogens, such as polycyclic aromatic hydrocarbons, were shown to be immunosuppressive in the animal models (Baldwin, 1973). Lastly, Good (1968) and his colleagues at the University of Minnesota observed that children born with inherited immune deficiencies had an increased risk of developing cancer, and others began to note that kidney transplant patients also had an increased risk of cancer while they were on immunosuppressive drugs (Vajdic *et al*., 1986; Chapman *et al*., 2006). More recently, with the advent of other immunosuppressive agents and other organ transplants, this increased risk has been corroborated and also observed in treatment with these agents for a variety of neoplastic and nonneoplastic disorders (Grulich *et al*., 2007). Additionally, acquired immunodeficiency disease syndrome (AIDS) is associated with an increased risk of developing cancers (Grulich *et al*., 2007). Kaposi's sarcoma was one of the original defining criteria for AIDS.

Although all these pieces of evidence suggested that there was immunosurveillance of neoplastic cells, in reality, it turns out that the immunosurveillance is of infectious organisms, just that some of these infections can produce cancer (Cohen, 1999b; Cohen *et al*., 1991; Schwartz, 1975). Thus, the tumors in immunosuppressed patients, whether inherited, secondary to chemotherapy for transplantation, malignancy, or autoimmune diseases, or in AIDS, occur only in certain tissues. These include B-cell lymphomas secondary to Epstein-Barr virus infections (or secondary to regeneration itself of the B-cell population), squamous cell tumors secondary to human papilloma virus, or Kaposi's sarcoma secondary to human herpes virus-8. There is some suggestion that there is an

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increased risk of liver cancer in patients with HBV or hepatitis C virus infections, also. However, there is not an increased risk of the more common tumors, such as cancer of the lung, colon, breast, or prostate or other tumors that do not have an infectious etiology.

Furthermore, the antigenicity of the mouse tumors was because of the presence of viruses that were used to induce the tumors or were activated in the tumorigenic process. Also, the doses necessary to produce immunosuppression, such as with polycyclic aromatic hydrocarbons, were actually higher than the doses necessary to produce tumors.

Based on the research over the past five decades, it is clear that the immunosurveillance is not of the malignant cells themselves but of the infections that can increase the risk of certain tumors.

Non–DNA-Reactive Carcinogens

In animal models, hormones and various treatments that affect the endocrine system frequently lead to an increased risk of tumors in the target population. This includes tumors of the rat thyroid, the rat testicular Leydig cells, and the endocrine cells of the stomach (which lead to carcinoid tumors). However, the only endocrine-related tumors in animal models that appear to be pertinent to humans are those associated with estrogen (Cohen, 2004). Estrogen is known to increase the risk of breast cancer and endometrial cancer in humans, and there is some suggestion that it also increases the risk of ovarian cancer. There is some evidence that estrogens also increase the risk of hepatocellular carcinomas (Preston-Martin *et al*., 1990), although more commonly the effect in humans is on the induction of hepatocellular adenomas, which are not pre-malignant lesions. The effect of estrogen on these target tissues is to increase the number of DNA replications in the target tissue pluripotential stem cell population (Preston-Martin *et al*., 1990). However, there is also evidence that estrogen can be metabolically activated to metabolites that react with DNA leading to adducts, which result in apurinic sites (Cavalieri *et al*., 2002). Whether the increase in cancer risk is due only to the increase in cell proliferation or because of a synergistic interaction between the DNA-reactive and cell proliferative effects remains a source of intense investigation. Nevertheless, estrogenic activity must be considered a risk factor for human cancer, at least of certain cell types.

As discussed above in detail, carcinogens can be classified as either DNA reactive or non-DNA reactive, relying on increased cell proliferation. The metabolic activation of DNA-reactive carcinogens has been studied extensively and includes classes of compounds such as the polycyclic aromatic hydrocarbons, aromatic amines, N-nitrosamines, related chemicals (such as hydrazines and N-nitroso-amides), aflatoxin, and numerous others. In each of these instances, exposures at high levels produce an increase in cell proliferation in the target tissue that greatly accentuates and accelerates the process. However, at lower exposure levels, the increase in incidence of tumors is proportionately considerably lower, giving a strikingly nonlinear dose-response, the so-called hockey stick dose-response curve. Extrapolating estimate of risk in humans for DNA-reactive carcinogens has traditionally assumed a linear, nonthreshold dose-response based to a large degree on the radiation model of mutagenesis and carcinogenesis (Calabrese, 2009) forming the basis for regulation of such chemicals. The shape of the dose-response for these chemicals, especially extending to low exposure levels, continues to be the focus of considerable heated debate.

Based on extensive investigations over the last five decades, there have also been a large number of chemicals that have been identified that increase the risk of cancer in animal models but are not DNA reactive (Cohen, 2004; Weisburger and Williams, 1981). In each of these instances studied in detail, the carcinogenic effect is because of an increase in cell proliferation. This can either be by a direct mitogenic effect (involving hormones and/or growth factors) or can be because of toxicity and regeneration. Toxicity can be produced either by induction of necrosis or by an increase in apoptosis in the target cell population. A decrease in cell deaths can occur by inhibiting apoptosis or by inhibiting differentiation. A combination of these effects can certainly occur. However, in each of these instances, the increase in cell proliferation represents a preneoplastic step in the process, which is essential for the ultimate development of tumors. In many instances, it is the actual toxicity that is of critical importance to human exposure, whereas the malignant consequence is either rare or the animal model of malignancy development is not relevant to humans. Attention to the actual toxicity that is involved is more relevant to human risk. A few examples that illustrate specific points are described below.

Sodium saccharin was shown in the 1970's to increase bladder cancer in rats when administered beginning at birth or earlier and continuing in the offspring generation for their lifetime (Cohen *et al*., 2008). The effect was greater in male rats than female rats, and mice were unaffected. Administration to monkeys beginning at birth and continuing for up to 24 years also did not increase cancer risk. Sodium saccharin in the rat leads to pronounced alterations in the composition of the various normal urinary constituents, leading to the formation of calcium phosphate–containing precipitate. This precipitate is cytotoxic to the urothelium, which leads to regenerative proliferation and ultimately to the development of a low incidence of tumors. The formation of this precipitate occurs more readily in male rats than in female rats predominantly because of the much higher level of protein in the male rat (α_{2u}-globulin). Mice do not develop the precipitate because of a much lower concentration of calcium and phosphate in the urine, and primates do not develop the precipitate because of the much lower density of constituents in the urine overall (osmolality of rodents is 1500–3000 mosmol, in contrast to humans below 400, and commonly below 250). Thus, the

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mechanism involved, calcium phosphate–containing precipitate is not relevant to humans (Cohen, 1999a; IARC WG, 1999). A similar precipitate occurs with the administration to rats of high doses of the sodium salt of any moderate to strong acid. Thus, calcium phosphate–containing precipitate and bladder tumors are induced following administration of comparably high doses of sodium ascorbate, chloride, bicarbonate, glutamate, and other sodium salts (Cohen *et al.*, 2000).

A related phenomenon has been identified in response to numerous chemicals leading to the production of urinary solids. One of these, melamine, leads to urinary calculi when administered to rats (IARC, 1999; IARC WG, 1999; Meek *et al.*, 2003). If the dose is insufficient to produce the calculi, there is no increase in proliferation or tumor formation. Thus, it is a high-dose phenomenon only, dependent on the physical property of solubility of the chemical. It was postulated that if humans were exposed environmentally to levels substantially lower than required for calculus formation, they would not have a tumorigenic response to melamine. However, in a recent unfortunate episode in China where baby formula was specifically adulterated with extraordinarily high levels of melamine, these children developed urinary calculi similar to what is seen in the rat, with urinary obstruction, hydronephrosis, and renal injury (Guan *et al.*, 2009; Meek *et al.*, 2003). Whether or not this would also lead to an increase of tumor risk can be argued based on the transient exposure of humans to these calculi. However, the reality is that the toxic effect, calculi, was similar between the rat and the human and actually at relatively comparable exposure levels. These calculi were composed predominantly of melamine with variable amounts of uric acid also present. Thus, in this instance, the risk assessment for humans involves an evaluation of the dose-response and identification of a threshold.

High doses of *d*-limonene produce an increased risk of renal tubular tumors in male rats (Hard and Whysner, 1994; IARC WG, 1999). The *d*-limonene is metabolized to an epoxide, which binds to a protein, α_{2u}-globulin, which is absorbed into the proximal tubular cells after passing through the glomerulus. In its bound form, the α_{2u}-globulin-*d*-limonene epoxide cannot be readily degraded so that there is an accumulation in lysosomes and ultimately an increase in cell death and consequent regeneration leading to tumors. This does not occur in female rats because they do not have substantial amounts of α_{2u}-globulin present. It also does not occur in mice, which have an analogous protein referred to as mouse urinary protein, but the epoxide does not bind to this protein and it does not lead to renal tubular cytotoxicity and regeneration. Humans do not have a protein that is comparable to α_{2u}-globulin to which the epoxide can bind. Thus, humans are not at risk for developing tumors in response to this mode of action.

These three chemicals all act by increasing cytotoxicity and regeneration. In contrast, there are numerous chemicals that have been identified in rats that produce a direct mitogenic effect such as increasing thyroid-stimulating hormone (TSH) by one means or another (Capen, 1998; Hill *et al.*, 1989; IARC WG, 1999). This leads to a direct mitogenic stimulus of the rat thyroid follicular cells and ultimately the development of benign and malignant tumors. Although humans have a similar feedback mechanism involving circulating thyroid hormones and TSH, the quantitative aspects are quite different. Humans have a circulating thyroid-binding globulin so that thyroid hormone is readily available if a stimulus occurs that leads to a decrease in circulating thyroid. In contrast, the rat does not have the circulating, readily available thyroid hormone, so its response is to increase TSH to stimulate the thyroid to produce more hormone by the follicular cells. Furthermore, the response to a hypothyroid stimulus in rats is to produce TSH, stimulating proliferation of follicular cells, leading to tumors. In contrast, hypothyroidism in humans leads to an increase in TSH, but this does not lead to an increase in follicular cell proliferation. It has been concluded that this mode of action in rats is not relevant to humans, based predominantly on a quantitative assessment of the process but also involving some qualitative issues. Again, the tumors arise from a process that leads to cell proliferation, and this process occurs early in the overall carcinogenicity of these chemicals. Epidemiologic investigations have not shown increased thyroid cancer associated with hypothyroidism, nor has it been shown to be related to chemical exposure, only radiation. Many chemicals have been shown to be toxic to the thyroid in animal models and in humans, but not thyroid carcinogens in humans.

Chloroform produces liver and renal tumors in rats and mice (Andersen *et al.*, 1998; Meek *et al.*, 2003). The mechanism involves metabolism to phosgene, which induces cytotoxicity and ultimately regenerative proliferation in both liver and kidney. Chloroform does not produce DNA reactivity, similar to all the other chemicals that have just been described regarding increased cell proliferation. It is thus clear that chloroform is carcinogenic only when the exposure is sufficiently high to produce cytotoxicity.

In humans, chloroform was once used as an anesthetic, and based on observations at those high levels of exposure, it was readily apparent that chloroform could produce hepatocellular toxicity as well as nephrotoxicity in humans. However, the effect was transient, disappearing once the anesthetic had been removed and the patient had the chance to recover from the toxicity. Whether such a high exposure level would produce tumors in humans in the liver and kidney is unknown, but the reality is that exposure to this chemical would not continue for a sustained period of time at high levels because of such toxicity. Because prolonged exposure appears to be required for carcinogenicity by these non–DNA-reactive carcinogens, it is unlikely that chloroform would pose a carcinogenic hazard even with short-term high-dose exposures, similar to the melamine story with calculi in infants. However, humans are also exposed to chloroform in

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chlorinated drinking water. The exposure in the drinking water is well below that which is anticipated to produce hepatocellular toxicity or nephrotoxicity and thus does not pose a carcinogenic hazard to humans. The actual toxicity assessment for chloroform is based on its cytotoxicity effects on the liver and kidney, not on the carcinogenicity, which is essentially a bystander effect resulting from the cytotoxicity.

There are numerous other examples that have been identified of carcinogenesis by non–DNA-reactive substances, and in many instances, the mode of action has been identified. These include the cytochrome P450 inducers, which appear to involve interaction with constitutive androstane receptor (CAR), which produce hepatocellular and thyroid tumors in rats and liver tumors in mice (Ross *et al*., 2009). Although humans have a similar receptor, activation of this receptor appears to lead to an increase in hepatocellular proliferation in rodents, whereas in human cells, this proliferative effect does not occur. A similar situation occurs with peroxisome proliferator-activated receptor alpha (PPARα) agonists (Gonzalez and Shah, 2008; Klaunig *et al*., 2003; Yang *et al*., 2008). Again, humans have a similar receptor, but increased cell proliferation is produced in rodents secondary to activation of the receptor, whereas that does not happen in humans.

Many non–DNA-reactive carcinogens have a mode of action involving interaction with specific cellular receptors, including nuclear receptors. This interaction with specific receptors, whether growth factor receptors or nuclear receptors, ultimately leads to increased cell proliferation. Examples include the CAR, PPARα, estrogen receptors, and numerous others. It has become apparent, however, that just identifying an interaction with a specific receptor does not define the relevance of rodent carcinogenesis to humans. Effects of activation of the receptor on downstream consequences can vary considerably between species, as well as species differences in coactivators and corepressors. For example, certain chemicals interact with CAR or PPARα receptors in rodents and humans, with similar metabolic consequences, such as activation of cytochrome P450s or peroxisomal enzymes, respectively. However, in rodents, there is also a proliferative response leading to cancer induction, but this does not occur in humans, and consequently, those chemicals are unlikely to be human carcinogens (Gonzalez and Shah, 2008; Klaunig *et al*., 2003; Ross *et al*., 2009).

In each of these instances and in many more, it is clear that the tumors arise secondary to some early occurring toxic event. By examining the mechanisms involved in the early event, rather than having to rely on a 2-year bioassay, considerable progress can be made more quickly in delineating the actual mechanisms involved with possible carcinogenesis.

Mode of Action and Human Relevance

During the past 15 years, regulatory agencies around the world have been evolving a framework to incorporate scientific understanding of the carcinogenic process into regulatory decision making. This has led to an evolving framework, starting with a process for evaluation of mode of action in animal models and then an assessment of its relevance to humans (Boobis *et al*., 2006, 2008; Meek *et al*., 2003; Seed *et al*., 2005; Sonich-Mullin *et al*., 2001). Activities to further develop this framework are continuing by incorporating various aspects of dose-response, exposure, cumulative risk, and other aspects involved in overall risk assessment. The important part of this process is the definition of mode of action for a given chemical in contrast to what is referred to as mechanism of action. A mode of action is a more generalized phenomenon without understanding the more detailed molecular events, which is referred to as the mechanism of action.

The mode of action for animal models is assessed utilizing a modification of the Bradford Hill's (1965) criteria originally developed for use in assessing causation in epidemiology investigations. This includes issues such as temporality, dose-response, biological plausibility, reproducibility of the data, and cohesiveness of the data. Once a mode of action is delineated in the animal model, there are then qualitative and quantitative assessments of this mode of action in humans. Frequently, there is no chemical-specific data available to assess this in humans, so surrogate systems need to be assessed. This can include *in vitro* models utilizing human cells and *in vivo* models utilizing so-called humanized tissues. These, unfortunately, are not definitive but provide considerable evidence that can be taken into account in an overall weight of evidence evaluation for a chemical. However, there is frequently information in humans that is highly relevant to the overall mode of action, even if chemical-specific data are not available. This can take into account, e.g., genetic abnormalities, such as inherited immunosuppressive disorders, as well as basic biochemical and physiological processes, such as thyroid hormone homeostasis and thyroid follicular proliferation in response to stimuli that are known to produce hypothyroidism. Epidemiologic findings, not only of the chemical but also of various physiological or metabolic dysfunctions like hypothyroidism, can provide valuable information.

Once the mode of action has been assessed, a qualitative assessment is then made based on the question: Can human relevance of the mode of action be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans? A concordance table comparing the effects of the key events in the animal model to the human situation is of considerable usefulness in this exercise. If the key events of the mode of action that are required for the animal model cannot occur in the human situation, such as formation of calcium phosphate–containing precipitate with sodium salts or an epoxide metabolite of *d*-limonene binding with α_{2u}-globulin, then the mode of action is not relevant to the human qualitatively and no cancer risk will occur. However, if the mode of action is relevant to humans qualitatively, then a quantitative assessment must be made. This incorporates differences in homeostatic control mechanisms, such as for thyroid carcinogenesis in the rat.

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Again, if the key events in the mode of action are not quantitatively relevant to humans, then there is no cancer risk for that chemical. After this analysis is completed, the data gaps, uncertainties, confidence in the analysis, and implication for the overall risk assessment are considered. Even if the mode of action for a given chemical is relevant to humans, the information gathered in the process of this evaluation can then be used in an overall risk assessment, including dose-response, mode of action, and modulating effects on the overall response.

Carcinogenicity Testing

As illustrated at the beginning of this article, many of the known human carcinogens have been identified by astute observations of clinical and occupational settings as well as occasionally by detailed epidemiologic evaluations. However, we would rather be able to identify which chemicals would be likely to increase our risk of cancer before such exposure occurs and prevent or reduce the exposure. Numerous methods have been developed to evaluate the potential carcinogenic risk to humans. The standard that has been used for more than four decades has been the 2-year bioassay in rats and mice. This evolved at the National Cancer Institute and then ultimately at the National Toxicology Program in the United States. This has formed the basis for screening of chemicals in the chemical, agrichemical, and pharmaceutical industries as well as more generally. However, this is a long, expensive, and resource-intensive exercise and is also fraught with sources of controversy regarding relevance of the mode of action to humans or the dose used in the study compared with human exposure. In addition, concordance of positivity in rats compared with mice is only 70–75% (Haseman and Huff, 1987) with target organ concordance even less (Maronpot *et al.*, 2004). Because of the high background incidences of certain tumors, such as liver, lung, vascular tumors, and lymphomas, in various mouse strains, along with other factors has led many to recommend not using the mouse for carcinogenicity testing (Alden *et al.*, 1996; Doe *et al.*, 2006). Short-term screens for chemical carcinogens have been sought to try to replace the 2-year bioassay. The first successful step in this process was the identification of the DNA reactivity of chemicals and the development of genotoxicity assays, particularly the Ames assay. This is widely used for screening of chemicals in combination with SAR considerations. However, as discussed above, this is useful only for detecting potential DNA-reactive carcinogens, and there are limitations to that as well.

A modification of the rodent bioassay has been developed over the last two decades utilizing transgenic and knockout mice (Cohen *et al.*, 2001). The ones that have been specifically evaluated for carcinogenicity screening include the p53 heterozygous knockout, the dual XPA/p53 knockout mouse, the rasH2 transgenic mouse, and the Tg.AC transgenic mouse for evaluation of dermal exposure (Pritchard *et al.*, 2003). Although some of these continue to be utilized for screening purposes, the difficulty arises when there is a positive tumor finding, as the mode of action is frequently unknown. Because of interpretation difficulties and sensitivity to irritation effects, the Tg.AC model has been essentially abandoned. The XPA/p53 model has also been abandoned because the observed effects appeared to be the same as those obtained with the p53 mouse. Of the remaining two models, the rasH2 seems to have gained more favor for general screening, whereas the p53 mouse model is used, somewhat erroneously, to assess genotoxicity. A major advance in utilizing these models is the considerably shorter period of time and decreased resources necessary to use them, but they have the same drawbacks as the 2-year bioassay in that they provide no information by themselves regarding mode of action or relevance to humans. This can only be gained by a shorter term assessment of specific key events that are likely to occur in a postulated mode of action.

Other test systems have been developed to try to identify the chemicals acting to increase carcinogenic risk by non–DNA-reactive processes. This has included *in vitro* inhibition of gap junction communication (Ruch and Trosko, 2001) as well as specific *in vivo* assays that utilize a combination of DNA-reactive and proliferative stimuli, such as the Ito's medium-term assay (Ito *et al.*, 2003). However, all these models have significant limitations in addressing the ultimate concern, human carcinogenic risk.

Based on the multistage model of carcinogenesis described above and the framework for mode of action and human relevance evaluation, another system has recently been proposed that utilizes shorter term exposure, assessing specifically the considerations for DNA reactivity, cell proliferation, estrogenicity, and immunosuppression (Cohen, 2004, 2010). Utilizing specific markers for various tissues, an initial screen for potential carcinogenicity in the rodent can be effective, with an extremely low likelihood of false negatives. Most importantly, utilizing mode of action analysis, short-term evaluations of detailed considerations of the mode of action, and dose-response, one can assess the overall human relevance of the mode of action and the likely exposure considerations for humans. A difficulty with this process is the high number of false positives that need to be evaluated in detail regarding mode of action. However, the data that are generated are directly applicable to evaluation of human risk.

With the explosion of genomics into biological research, it has become a focal point for considerable investigation to utilize such systems for screening for chemical carcinogenicity and other toxin endpoints (Ellinger-Ziegelbauer *et al.*, 2008; Nie *et al.*, 2006; Rotroff *et al.*, 2010; Thomas *et al.*, 2007). Initial results are encouraging regarding DNA-reactive chemicals and for identifying specific modes of action for known non–DNA-reactive carcinogens. It remains unclear whether it provides a useful screening method in general, especially in detecting chemicals that might act by a mode of action not yet identified. A major drawback of these approaches is the ultimate need to examine by genomic screening a very large

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number of tissues. Most of the work done to date has involved the liver. In contrast to rodents, where liver is the most common target site for carcinogenicity of chemicals, liver cancer in humans is generally not related to chemical exposure except in unusual instances such as aflatoxin and ethanol. Based on previous screening methodologies, it is very likely that screening of the liver will be useful in detecting rodent liver carcinogens but probably will not be of much use in detecting carcinogens active at other target sites. It still leaves the question of human relevance.

Transgenic and knockout models have generally not been widely used for screening purposes, and mode of action analysis if a positivity is detected in these assays is a difficult exercise. However, specific, targeted knockout, and transgenic models can be extremely useful in evaluating specific modes of action. For example, cytochrome P450 (CYP) CYP2E1 is essential for the oxidative metabolism of chloroform to phosgene (Andersen *et al*., 1998; Meek *et al*., 2003). Mice specifically knocked out for this gene are unable to oxidatively metabolize chloroform and do not develop liver or kidney toxicity, prerequisites for the ultimate development of tumors. Similarly, *in vitro* models as well as humanized mouse and rat models can be of considerable usefulness in evaluating specific modes of action for a given chemical (Yang *et al*., 2008). However, as described above, all these have limitations. It is critical that all the information available be incorporated into any assessment of the mode of action and its relevance to humans, as well as the dose-response.

For toxicology testing, including testing for carcinogenicity, practical considerations need to be incorporated. Several suggestions have been made to begin modifying regulatory decision making so that the tens of thousands of chemicals in commerce and in the environment can be addressed. For chemicals that we are exposed to at very low amounts, the toxicological threshold of concern approach is being used in certain circumstances, such as for flavors and food and drug contaminants (Munro *et al*., 1996). Modifications to the testing of agrichemicals have been recommended and are being assessed (Carmichael *et al*., 2006; Doe *et al*., 2006). Other approaches for specific classes of chemicals are being evaluated. Numerous high-throughput approaches have been developed as potential screening tools for the many thousands of chemicals that need to be evaluated, such as the TOXCAST project of the EPA (Kavlock and Dix, 2010).

A recent publication of the National Research Council, Toxicity Testing in the 21st Century: A Vision and a Strategy, was published in 2007 (NRC, 2007), and describes a vision for toxicity testing in the future. This is based on a biological systems approach to perturbations of the complex interactive pathways controlling cellular functions and utilizing *in vitro* cellular model systems, ultimately utilizing human cells for an analysis of potential adverse reactions in humans.

Although the vision of the NRC report is laudable and worthwhile pursuing, we are far from being able to implement it at this time. We do not have available appropriate cell lines that appropriately mimic the response *in vivo*, all the metabolic activation and inactivation processes are not preserved *in vitro*, and current *in vitro* approaches are incapable of addressing the common occurrence of organ interactions that are involved in many toxic endpoints. Quantitative extrapolation from *in vitro* to *in vivo* is even more precarious without first examining toxicokinetics *in vivo*. In addition, we do not yet know the intricacies of the various pathways as they pertain to modulating effects and distinguishing between adaptive and adverse. Fortunately, there are many talented scientists investigating these issues, and progress is being made. The danger is adopting some of these screening tests before we adequately understand their relevance and implications. As has been suggested (MacDonald and Robertson, 2009), it is important to focus on appropriate testing based on sound science rather than accepting a test as real, just another example of an Ames test approach. It is particularly easy to be seduced by the spectacular technologies now available, losing sight of the basic biology. It is particularly critical as we go forward to focus on the ultimate question, is it toxic to humans, animals, or the environment and, if so, at what exposure level? Toxicology began with the simple premise that all substances are poisons, but the dose makes the poison (Paracelsus, see Gallo, 2008). The technologies are tools to address the science.

CONCLUSION

We have come a long way since Sir Percival Pott identified the association of scrotal cancer in chimney sweeps, but the major questions that he raised still require continued investigation with the development of better methods. The focus needs to be on the ultimate question of relevance to human risk rather than accepting findings in model systems as sacrosanct. The issues of dose-response, linearity, threshold, and exposure, in addition to the human relevance issue, remain at the heart of our concerns. We need to remember that approximately one-fourth to one-third of individuals in the United States will develop cancer some time in their lifetime. Eliminating causes is essential for decreasing risk. However, even identifying the causes does not guarantee that we are able to implement preventive measures, as can be all too readily seen with cigarette smoking, obesity, and sexually transmitted diseases.

REFERENCES

Alden, C. L., Smith, P. F., Piper, C. E., and Brej, L. (1996). A critical appraisal of the value of the mouse cancer bioassay in safety assessment. *Toxicol. Pathol.* **24,** 722–725.

Ames, B. N., Durston, W. E., Yamasaki, E., and Lee, F. D. (1973). Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl. Acad. Sci. U.S.A.* **70,** 2281–2285.

Downloaded from toxsci.oxfordjournals.org at Masarykova univerzita on February 23, 2011

Andersen, M., Brusick, D., Cohen, S., Dragan, Y., Frederick, C., Goodman, J. I., Hard, G., Meek, B., and O'Flaherty, E. J. (1998). U.S. Environmental Protection Agency's revised cancer guidelines for carcinogen risk assessment. *Toxicol. Appl. Pharmacol.* **153,** 133–136.

Armitage, P., and Doll, R. (1954). The age distribution of cancer and a multi-stage theory of carcinogenesis. *Br. J. Cancer* **8,** 1–12.

Baldwin, R. W. (1973). Immunological aspects of chemical carcinogenesis. *Adv. Cancer Res.* **18,** 1–75.

Berenblum, I., and Shubik, P. (1947). A new quantitative approach to the study of stages of carcinogenesis in the mouse's skin. *Br. J. Cancer* **1,** 383–391.

Boddy, A. V., and Ratain, M. J. (1997). Pharmacogenetics in cancer etiology and chemotherapy. *Clin. Cancer Res.* **3,** 1025–1030.

Boobis, A. R., Cohen, S. M., Dellarco, V., McGregor, D., Meek, M. E., Vickers, C., Willcocks, D., and Farland, W. (2006). IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36,** 781–792.

Boobis, A. R., Doe, J. E., Heinrich-Hirsch, B., Meek, M. E., Munn, S., Ruchirawat, M., Schlatter, J., Seed, J., and Vickers, C. (2008). IPCS framework for analyzing the relevance of a noncancer mode of action for humans. *Crit. Rev. Toxicol.* **38,** 87–96.

Boutwell, R. K. (1964). Some biological aspects of skin carcinogenesis. *Progr. Exp. Tumor Res.* **4,** 207–250.

Boveri, T. (1914). In *Zur Frage der Enstehung Maligner Tumoren.* Verlag von Gustav Fischer, Jena, Germany.

Bryan, G. T. (1969). Role of tryptophan metabolites in urinary bladder cancer. *Am. Ind. Hyg. Assoc. J.* **30,** 27–34.

Burnet, M. F. (1957). Cancer—a biological approach. IV. Practical applications. *Br. Med. J.* **1,** 844–847.

Burnet, M. F. (1964). Immunologic factors in the process of carcinogenesis. *Br. Med. Bull.* **20,** 154–158.

Calabrese, E. J. (2009). The road to linearity: why linearity at low doses became the basis for carcinogen risk assessment. *Arch. Toxicol.* **83,** 203–225.

Capen, C. C. (1998). Correlation of mechanistic data and histopathology in the evaluation of selected toxic endpoints of the endocrine system. *Toxicol. Lett.* **102,** 405–409.

Carmichael, N. G., Barton, H. A., Boobis, A. R., Cooper, R. L., Dellarco, V. L., Doerrer, N. G., Fenner-Crisp, P. A., Doe, J. E., Lamb, I. V., JC., *et al.* (2006). Agricultural chemical safety assessment: a multisector approach to the modernization of human safety requirements. *Crit. Rev. Toxicol.* **36,** 1–7.

Cavalieri, E. L., Li, K. M., Balu, N., Saeed, M., Devanesan, P., Higginbotham, S., Zhao, J., Gross, M. L., and Rogan, E. G. (2002). Catechol ortho-quinones: the electrophilic compounds that form depurinating DNA adducts and could initiate cancer and other diseases. *Carcinogenesis* **23,** 1071–1077.

Chapman, J. R., Webster, A. C., Kaldor, J. M., and Grulich, A. E. (2006). Cancer incidence before and after kidney transplantation. *JAMA* **296,** 2823–2831.

Chuang, S. C., La Vecchia, C., and Boffetta, P. (2009). Liver cancer: descriptive epidemiology and risk factors other than HBV and HCV infection. *Cancer Lett.* **286,** 9–14.

Clayson, D. B., and Cooper, E. H. (1970). Cancer of the urinary tract. In *Advances in Cancer Research.* (G. Klein and S. Weinhouse, Eds.), Vol. 13, pp. 271–381. Academic Press, Inc., New York, NY.

Cleaver, J. E., Charles, W. C., McDowell, M. L., Sadinski, W. J., and Mitchell, D. L. (1975). Overexpression of the XPA repair gene increases resistance to ultraviolet radiation in human cells by selective repair of DNA damage. *Cancer Res.* **55,** 6152–6160.

Cohen, S. M. (1991). Analysis of modifying factors in chemical carcinogenesis. In *Modification of Tumor Development of Rodents, Progress in Experimental Tumor Research.* (N. Ito, Ed.), Vol. 33, pp. 21–40. Karger and Basel, New York, NY.

Cohen, S. M. (1998a). Cell proliferation and carcinogenesis. *Drug Metab. Rev.* **30,** 339–357.

Cohen, S. M. (1998b). Cell proliferation in the evaluation of carcinogenic risk and the inadequacies of the initiation-promotion model. *Int. J. Toxicol.* **17,** 129–142.

Cohen, S. M. (1999a). Calcium phosphate-containing urinary precipitate in rat urinary bladder carcinogenesis. International Agency for Research on Cancer. *IARC Sci. Publ.* **147,** 175–189.

Cohen, S. M. (1999b). Infection, cell proliferation, and malignancy. In *Microbes and Malignancy: Infection as a Cause of Cancer.* (J. Parsonnet and S. Horning, Eds.), pp. 89–106. Oxford University Press, New York, NY.

Cohen, S. M. (2004). Human carcinogenic risk evaluation: an alternative approach to the two-year rodent bioassay. *Toxicol. Sci.* **80,** 225–229.

Cohen, S. M. (2010). Evaluation of possible carcinogenic risk to humans based on liver tumors in rodent assays: the two year bioassay is no longer necessary. *Toxicol. Pathol.* **38,** 487–501.

Cohen, S. M., Arnold, L. L., Cano, M., Ito, M., Garland, E. M., and Shaw, R. A. (2000). Calcium phosphate-containing precipitate and the carcinogenicity of sodium salts in rats. *Carcinogenesis* **21,** 783–792.

Cohen, S. M., Arnold, L. L., and Emerson, J. L. (2008). Safety of saccharin. *Agro Food Ind. Hi-Tech.* **19,** 26–29.

Cohen, S. M., Boobis, A. R., Meek, M. E., Preston, R. J., and McGregor, D. B. (2006). 4-Aminobiphenyl and DNA reactivity: case study within the context of the 2006 IPCS human relevance framework for analysis of a cancer mode of action for humans. *Crit. Rev. Toxicol.* **36,** 803–819.

Cohen, S. M., and Ellwein, L. B. (1990a). Cell proliferation in carcinogenesis. *Science* **249,** 1007–1011.

Cohen, S. M., and Ellwein, L. B. (1990b). Proliferative and genotoxic cellular effects in 2-acetylaminofluorene bladder and liver carcinogenesis: biological modeling of the ED_{01} study. *Toxicol. Appl. Pharmacol.* **104,** 79–93.

Cohen, S. M., and Ellwein, L. B. (1991). Genetic errors, cell proliferation, and carcinogenesis. *Cancer Res.* **51,** 6493–6505.

Cohen, S. M., Purtilo, D. T., and Ellwein, L. B. (1991). Pivotal role of increased cell proliferation in human carcinogenesis. *Modern Pathol.* **4,** 371–382.

Cohen, S. M., Robinson, D., and MacDonald, J. (2001). Alternative models for carcinogenicity testing. *Toxicol. Sci.* **64,** 14–19.

Cooper, E. H., Frank, G. L., and Wright, D. H. (1966). Cell proliferation in Burkitt tumours. *Eur. J. Cancer* **2,** 377–384.

Creech, J., Jr., and Johnson, M. (1974). Angiosarcoma of liver in the manufacture of polyvinyl chloride. *J. Occup. Med.* **16,** 150–151.

DeBaun, J. R., Miller, E. C., and Miller, J. A. (1970). N-Hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein(methion-S-yl) binding in rat liver. *Cancer Res.* **30,** 577–595.

Doe, J. E., Boobis, A. R., Blacker, A., Dellarco, V., Doerrer, N. G., Franklin, C., Goodman, J. I., Kronenberg, J. M., Lewis, R., McConnell, E. E., *et al.* (2006). A tiered approach to systemic toxicity testing for agricultural chemical safety assessment. *Crit. Rev. Toxicol.* **36,** 37–69.

Dragan, Y. P., Sargent, L., Xu, Y. D., Xu, Y. H., and Pitot, H. C. (1993). The initiation-promotion-progression model of rat hepatocarcinogenesis. *Proc. Soc. Exp. Biol. Med.* **202,** 16–24.

Ellinger-Ziegelbauer, H., Gmuender, H., Bandenburg, A., and Ahr, H. J. (2008). Prediction of a carcinogenic potential of rat hepatocarcinogens using toxicogenomics analysis of short-term in vivo studies. *Mutat. Res.* **637,** 23–39.

Downloaded from toxsci.oxfordjournals.org at Masarykova univerzita on February 23, 2011

Gallo, M. A. (2008). History and scope of toxicology. In *Toxicology. The Basic Science of Poisons*, 7th ed. (C. D. Klaassen, Ed.), pp. 3–10. McGraw Hill Medical, New York, NY.

Gee, P., Maron, D. M., and Ames, B. N. (1994). Detection and classification of mutagens: a set of base-specific Salmonella tester strains. *Proc. Natl. Acad. Sci. U.S.A.* **91,** 11606–11610.

Gonzalez, F. J., and Shah, Y. M. (2008). PPARalpha: mechanism of species differences and hepatocarcinogenesis of peroxisome proliferators. *Toxicology* **246,** 2–8.

Good, R. A. (1968). Experiments of nature in immunobiology. *N. Engl. J. Med.* **279,** 1344–1345.

Greenfield, R. E., Ellwein, L. B., and Cohen, S. M. (1984). A general probabilistic model of carcinogenesis: analysis of experimental urinary bladder cancer. *Carcinogenesis* **5,** 437–445.

Grulich, A. E., van Leeuwen, M. T., Falster, M. O., and Vajdic, C. M. (2007). Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet* **370,** 59–67.

Guan, N., Fan, Q., Ding, J., Zhao, Y., Lu, J., Ai, Y., Xu, G., Zhu, S., Yao, C., Jiang, L., *et al.* (2009). Melamine-contaminated powdered formula and urolithiasis in young children. *N. Engl. J. Med.* **360,** 1067–1074.

Guengerich, F. P. (2000). Metabolism of chemical carcinogens. *Carcinogenesis* **21,** 345–351.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999). Creation of human tumour cells with defined genetic elements. *Nature* **400,** 464–467.

Hard, G. C., and Whysner, J. (1994). Risk assessment of *d*-limonene: an example of male rat-specific renal tumorigens. *Crit. Rev. Toxicol.* **24,** 231–254.

Hart, R. W., Keenan, K., Turturro, A., Abdo, K. M., Leakey, J., and Lyn-Cook, B. (1995). Caloric restriction and toxicity. *Fund. Appl. Toxicol.* **25,** 184–195.

Haseman, J. D., and Huff, J. E. (1987). Species correlation in long-term carcinogenicity studies. *Cancer Lett.* **34,** 125–132.

Hein, D. W. (2006). N-acetyltransferase 2 genetic polymorphism: effects of carcinogen and haplotype on urinary bladder cancer risk. *Oncogene* **25,** 1649–1658.

Hennekens, C. H., Buring, J. E., Manson, J. E., Stampfer, M., Rosner, B., Cook, N. R., Belanger, C., LaMotte, F., Gaziano, J. M., Ridker, P. M., *et al.* (1996). Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *N. Engl. J. Med.* **334,** 1145–1149.

Hill, A. B. (1965). The environment and disease: association or causation? *Proc. R. Soc. Med.* **58,** 295–300.

Hill, J. (1761). Cautions against the immoderate use of snuff . (R. Baldwin and J. Jackson, Eds.), London, UK.

Hill, R. N., Erdreich, L. S., Paynter, O. E., Roberts, P. A., Rosenthal, S. L., and Wilkinson, C. F. (1989). Thyroid follicular cell carcinogenesis. *Fundam. Appl. Toxicol.* **12,** 629–697.

Hoeijmakers, J. H. (2009). DNA damage, aging, and cancer. *N. Engl. J. Med.* **361,** 1475–1485.

Hueper, W. C., Wiley, F. H., and Wolfe, H. D. (1938). Experimental production of bladder tumors in dogs by administration of beta-naphthylamine. *J. Ind. Hyg. Toxicol.* **20,** 46–84.

International Agency for Research on Cancer (IARC). (1999). Saccharin and its salts. Some chemicals that cause tumours of the kidney or urinary bladder in rodents and some other substances. *IARC Monogr. Eval. Carcinog. Risks Hum.* **73,** 517–624.

IARC Working Group (IARC WG). (1999). Consensus report. International Agency for Research on Cancer. *IARC Sci. Publ.* **147,** 1–32.

Ito, N., Tamano, S., and Shirai, T. (2003). A medium-term rat liver bioassay for rapid in vivo detection of carcinogenic potential of chemicals. *Cancer Sci.* **94,** 3–8.

Kavlock, R., and Dix, D. (2010). Computational toxicology as implemented by the U.S. EPA: providing high throughput decision support tools for screening and assessing chemical exposure, hazard and risk. *J. Toxicol. Environ. Health B Crit. Rev.* **13,** 197–217.

Kennaway, E., and Hieger, I. (1930). Carcinogenic substances and their fluorescence spectra. *Br. Med. J.* **1,** 1044–1046.

Kinosita, R. (1936). Research on the carcinogenesis of the various chemical substances. *Gann* **30,** 423–426.

Kirkland, D., Pfuhler, S., Tweats, D., Aardema, M., Corvi, R., Darroudi, F., Elhajouji, A., Glatt, H., Hastwell, P., Hayashi, M., *et al.* (2007). How to reduce false positive results when undertaking in vitro genotoxicity testing and thus avoid unnecessary follow-up animal tests: report of an ECVAM Workshop. *Mutat. Res.* **628,** 31–55.

Klaunig, J. E., Babich, M. A., Baetcke, K. P., Cook, J. C., Corton, J. C., David, R. M., DeLuca, J. G., Lai, D. Y., McKee, R. H., Peters, J. M., *et al.* (2003). PPARalpha agonist-induced rodent tumors: modes of action and human relevance. *Crit. Rev. Toxicol.* **33,** 655–780.

Klein, G., and Klein, E. (1986). Conditioned tumorigenicity of activated oncogenes. *Cancer Res.* **46,** 3211–3224.

Knudson, A. G. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. U.S.A.* **68,** 820–823.

Knudson, A. G. (1993). Antioncogenes and human cancer. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 10914–10921.

Kodama, M., and Kodama, T. (1998). Comparative epidemiology of cancers of the testis, lung, bladder and stomach with special reference to the possible implication of environmental hormones in the recent risk changes of the 4 neoplasia types. *Int. J. Mol. Med.* **2,** 705–714.

Kripke, M. L., and Borsos, T. (1974). Editorial: Immune surveillance revisited. *J. Natl. Cancer Inst* **52,** 1393–1395.

Kritchevsky, D. (1999). Caloric restriction and experimental carcinogenesis. *Toxicol. Sci.* **52**(Suppl. 2), 13–16.

Lambert, I., Singer, T., Boucher, S. E., and Douglas, G. R. (2005). Detailed review of transgenic rodent mutation assays. *Mutat. Res.* **590,** 1–280.

Lower, G. M., Jr., and Bryan, G. T. (1973). Enzymatic N-acetylation of carcinogenic aromatic amines by liver cytosol of species displaying different organ susceptibilities. *Biochem. Pharmacol.* **22,** 1581–1588.

Lu, M. H., Hinson, W. G., Turturro, A., Sheldon, W. G., and Hart, R. W. (1993). Cell proliferation by cell cycle analysis in young and old dietary restricted mice. *Mech. Ageing Dev.* **68,** 151–162.

Lutz, W. K., and Schlatter, J. (1992). Chemical carcinogens and overnutrition in diet-related cancer. *Carcinogenesis* **13,** 2211–2216.

MacDonald, J. S., and Robertson, R. T. (2009). Toxicity testing in the 21st century: a view from the pharmaceutical industry. *Toxicol. Sci.* **110,** 40–46.

Marnett, L. J. (2009). The COXIB experience: a look in the rearview mirror. *Annu. Rev. Pharmacol. Toxicol.* **49,** 265–290.

Maronpot, R. R., Flake, G., and Huff, J. (2004). Relevance of animal carcinogenesis findings to human cancer predictions and prevention. *Toxicol. Pathol.* **32**(Suppl. 1), 40–48.

McCann, J., Spingarn, N. E., Kobori, J., and Ames, B. N. (1975). Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 979–983.

Meek, M. E., (Bette), Bucher, J. R., Cohen, S. M., Dellarco, V., Hill, R. N., Lehman-McKeeman, L. D., and Longfellow, D. G. (2003). Pastoor, T., Seed, J., and Patton, D. E. (2003). A framework for human relevance analysis of information on carcinogenic modes of action. *Crit. Rev. Toxicol.* **33,** 591–653.

Downloaded from toxsci.oxfordjournals.org at Masarykova univerzita on February 23, 2011

Miller, J. A., and Miller, E. C. (1977). Ultimate chemical carcinogens as reactive mutagenic electrophiles. In *Origins of Human Cancer* (H. H. Hiatt, Ed.), pp. 605–627. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mitchell, S. C., and Smith, R. L. (2010). A physiological role for flavin-containing monooxygenase (FMO3) in humans? *Xenobiotica* **40,** 301–305.

Moolgavkar, S. H., and Knudson, A. G. J. (1981). Mutation and cancer: a model for human carcinogenesis. *J. Natl. Cancer Inst.* **66,** 1037–1052.

Munro, I. C., Ford, R. A., Kennepohl, E., and Sprenger, J. G. (1996). Correlation of structural class with no-observed-effect levels: a proposal for establishing a threshold of concern. *Food Chem. Toxicol.* **34,** 829–867.

Murasaki, G., and Cohen, S. M. (1983). Co-carcinogenicity of sodium saccharin and N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide for the urinary bladder. *Carcinogenesis* **4,** 97–99.

National Research Council (NRC). (2007). *Toxicity Testing in the 21st Century: A Vision and a Strategy*. National Academies Press, Washington, DC.

Nie, A. Y., McMillian, M., Parker, J. B., Leone, A., Bryant, S., Yieh, L., Bittner, A., Nelson, J., Carmen, A., Wan, J., *et al.* (2006). Predictive toxicogenomics approaches reveal underlying molecular mechanisms of nongenotoxic carcinogenicity. *Mol. Carcinog.* **45,** 914–933.

Phillips, D. H., Farmer, P. B., Beland, F. A., Nath, R. G., Poirier, M. C., Reddy, M. V., and Turteltaub, K. W. (2000). Methods of DNA adduct determination and their application to testing compounds for genotoxicity. *Environ. Mol. Mutagen.* **35,** 222–233.

Poirier, M. C., Beland, F. A., Deal, F. H., and Swenberg, J. A. (1989). DNA adduct formation and removal in specific liver cell populations during chronic dietary administration of 2-acetylaminofluorene. *Carcinogenesis* **10,** 1143–1145.

Pott, P. (1775). Chirurgical Observations Relative to the Cataract. The Polypus of the Nose, the Cancer of the Scrotum, the Different Kinds of Ruptures, and the Mortification of the Toes and Feet. Chapter III. Hawes, W. Clarke, and R. Collins, London, UK. pp. 60–68.

Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A., and Henderson, B. E. (1990). Increased cell division as a cause of human cancer. *Cancer Res.* **50,** 7415–7421.

Prichard, J. B., French, J. E., Davis, B. J., and Haseman, J. K. (2003). The role of transgenic mouse models in carcinogen identification. *Environ. Health Perspec.* **111,** 444–454.

Rehn, L. (1895). Blasengeschwülste be: Fuchsin-arbeitern. *Arch. Klin. Chir.* **50,** 588–600.

Ross, P. K., Woods, C. G., Bradford, B. U., Kosyk, O., Gatti, D. M., Cunningham, M. L., and Rusyn, I. (2009). Time-course comparison of xenobiotic activators of CAR and PPARalpha in mouse liver. *Toxicol. Appl. Pharmacol.* **235,** 199–207.

Rotroff, D. M., Wetmore, B. A., Dix, D. J., Ferguson, S. S., Clewell, H. J., Houck, K. A., LeCluyse, E. L., Andersen, M. E., Judson, R. S., Smith, C. M., *et al.* (2010). Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. *Toxicol. Sci.* **117,** 348–358.

Ruch, R. J., and Trosko, J. E. (2001). Gap-junction communication in chemical carcinogenesis. *Drug Metab. Rev.* **33,** 117–121.

Schwartz, R. S. (1895). Another look at immunologic surveillance. *N. Engl. J. Med.* **293,** 181–184.

Seed, J., Carney, E. W., Corley, R. A., Crofton, K. M., DeSesso, J. M., Foster, P. M. D., Kavlock, R., Kimmel, G., Klaunig, J., Meek, M. E., *et al.* (2005). Overview: Using mode of action and life stage information to evaluate the human relevance of animal toxicity data. *Crit Rev. Toxicol.* **35,** 663–672.

Seifried, H. E., Seifried, R. M., Clarke, J. J., Junghans, T. B., and San, R. H. (2006). A compilation of two decades of mutagenicity test results with the Ames Salmonella typhimurium and L5178Y mouse lymphoma cell mutation assays. *Chem. Res. Toxicol.* **19,** 627–644.

Shin, A., Shrubsole, M. J., Rice, J. M., Cai, Q., Doll, M. A., Long, J., Smalley, W. E., Shyr, Y., Sinha, R., Ness, R. M., *et al.* (2008). Meat intake, heterocyclic amine exposure, and metabolizing enzyme polymorphisms in relation to colorectal polyp risk. *Cancer Epidemiol. Biomarkers Prev.* **17,** 320–329.

Society of Toxicology (SOT). (1981). Re-examination of the ED_{01} study—overview. *Fundam. Appl. Toxicol.* **1,** 28–63.

Sonich-Mullin, C., Fielder, R., Wiltse, J., Baetcke, K., Dempsey, J., Fenner-Crisp, P., Grant, D., Hartley, M., Knaap, A., Kroese, D., Mangelsdorf, I., Meek, E., Rice, J. M., and Younes, M. (2001). IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul. Toxicol. Pharmacol.* **34,** 146–152.

Tannenbaum, A. (1940). The initiation and growth of tumors. Introduction. Effects of undernutrition. *Am. J. Cancer* **38,** 335–350.

Thomas, L. (1959). Discussion. In *Cellular and Humoral Aspects of Hypersensitive States* (H. S. Lawrence, Ed.), p. 529. Hoeber-Harper, New York, NY.

Thomas, R. S., Pluta, L., Yang, L., and Halsey, T. A. (2007). Application of genomic biomarkers to predict increased lung tumor incidence in 2-year rodent cancer bioassays. *Toxicol. Sci.* **97,** 55–64.

Tong, W., Welsh, W. J., Shi, L., Fang, H., and Perkins, R. (2003). Structure-activity relationship approaches and applications. *Environ. Toxicol. Chem.* **22,** 1680–1695.

Vajdic, C. M., McDonald, S. P., McCredie, M. R., van Leeuwen, M. T., Stewart, J. H., Law, M., Van Kaick, G., Wesch, H., Lührs, H., and Liebermann, D. (1986). Radiation-induced primary liver tumors in "thorotrast patients". *Rec. Results Cancer Res.* **100,** 16–22.

Vastag, B. (2009). Nutrients for prevention: negative trials send researchers back to drawing board. *J. Natl. Cancer Inst.* **101,** 446–448; 451.

Watanabe, S., Brown, C., and Young, J. (1986). Cumulative incidence rates for Hodgkin's disease and other hematologic malignancies, with special reference to age-related carcinogenesis. *Jpn. J. Cancer Res.* **77,** 743–749.

Wattenberg, L. W. (1985). Chemoprevention of cancer. *Cancer Res.* **45,** 1–8.

Weisburger, J. H., and Williams, G. M. (1981). Carcinogen testing: current problems and new approaches. *Science* **214,** 401–407.

Whysner, J., Ross, P. M., and Williams, G. M. (1996). Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation, and tumor promotion. *Pharmacol. Ther.* **71,** 153–191.

Wood, L. D., Parsons, D. W., Jones, S., Lin, J., Sjöblom, T., Leary, R. J., Shen, D., Boca, S. M., Barber, T., Ptak, J., *et al.* (1996). Phenobarbital mechanistic data and risk assessment: enzyme induction, enhanced cell proliferation, and tumor promotion. *Pharmacol. Ther.* **71,** 153–191.

Wu, W. K., Sung, J. J., Lee, C. W., Yu, J., and Cho, C. H. (2010). Cyclooxygenase-2 in tumorigenesis of gastrointestinal cancers: an update on the molecular mechanisms. *Cancer Lett.* **295,** 7–16.

Yamagiwa, K., and Ichikawa, K. (1915). Experimentelle studie über die pathogenese der Epithelialgeschwülste. *Mitt. Med. Fak. Kaiserl Univ. Tokio* **15,** 295–344.

Yang, Q., Nagano, T., Shah, Y., Cheung, C., Ito, S., and Gonzalez, F. J. (2008). The PPAR alpha-humanized mouse: a model to investigate species differences in liver toxicity mediated by PPAR alpha. *Toxicol. Sci.* **101,** 132–139.

Yoshida, T. (1933). Über die serienweise Verfolgung der Veränderungen der Leber der experimentellen Hepatomerzeugung durch o-aminoazotuol. *Trans. Jpn. Pathol. Soc.* **23,** 636–638.

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