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Review

Thresholds in Genotoxicity and Carcinogenicity: Urinary Bladder Carcinogenesis[1]

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Cancer is due to multiple alterations to DNA. Chemicals can increase the cancer risk by directly damaging DNA (DNA reactivity) or by increasing cell proliferation (DNA replications), increasing the number of opportunities for spontaneous DNA damage. Genotoxicity is a more comprehensive term than DNA reactivity. Many of the mechanisms of genotoxicity, such as clastogenicity, inhibition of DNA repair, or damage to the mitotic apparatus, produce DNA damage indirectly. These non-DNA reactive mechanisms involve interactions with proteins and mechanistically are threshold phenomena. 2-Acetylaminofluorene (AAF) is DNA reactive. Its dose response for urinary bladder DNA adduct formation is linear, whereas the tumor response is non-linear. Non-linearity is at the dose at which increased cell proliferation occurs, related to the threshold phenomenon of cytotoxicity. Non-linearity for DNA reactive carcinogens can also be produced by changes in metabolic processes of activation and/or deactivation due to saturable kinetics. Arsenic produces bladder cancer with a non-linear dose response in animal models and humans. Genotoxicity of arsenic occurs secondarily to indirect mechanisms, not DNA reactivity, it has a non-linear dose response, and the genotoxic mechanism appears to have a threshold, occurring only at doses in excess of toxic concentrations. Numerous non-genotoxic agents have been identified as bladder carcinogens in rodent models, most acting by inducing cytotoxicity with regenerative proliferation. Cytotoxicity can be produced by formation of urinary solids or by urinary reactive chemicals. Urinary solids are a defined threshold phenomenon based on the physical-chemical property of solubility. Likewise, chemical induction of cytotoxicity is a known threshold phenomenon. Non-genotoxic chemicals have a threshold dose response with respect to carcinogenesis, as do most genotoxic agents. DNA reactive chemicals have a non-linear dose response.

Key words: sodium saccharin, calculi, arsenic, acetylaminofluorene, genotoxicity

Numerous chemicals have been identified as causing cancer in humans. To avoid the release of additional chemical carcinogens into the environment, numerous screening tests have been developed to try to identify such chemicals so that they can be avoided. Nevertheless, numerous chemical carcinogens remain in the environment, although most are present at extremely low levels of exposure. The question arises as to whether any of these low levels of exposure pose an actual cancer risk to humans, or is there a level of exposure below which there is no risk, a so-called threshold.

In this presentation I will first present a theoretical framework of carcinogenesis on which to evaluate this question, and then present several examples illustrating various aspects of this question. I am focusing on urinary bladder carcinogens since there is considerable information regarding chemicals that can induce bladder cancer either in experimental models or in humans (1). Numerous specific chemicals and mixtures have been identified as human bladder carcinogens, and much is known about their metabolic activation processes, kinetics, and dynamics. In addition, numerous extensive epidemiologic investigations have been performed to investigate various aspects of chemical carcinogenesis with respect to the urinary bladder.

Chemicals have been known to be responsible for the induction of bladder cancer since the first original observation by Rehn in 1895 of an association between exposure of workers in the aniline dye industry in Germany and the development of bladder cancer (1,2). Research into this problem eventually led to the identification of aromatic amines as the chemicals responsible for carcinogenicity (2). Extensive research into the metabolic processes of aromatic amines led to the development of the reactive electrophile theory of carcinogenesis proposed by the Millers (3). Numerous other chemicals have been identified as human bladder carcinogens, including phosphoramide mustards and ar-

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senic, but the most important causative agent for bladder cancer in developed countries is cigarette smoking (1). Cigarette smoke contains large quantities of various aromatic amines, but especially 4-aminobiphenyl. In addition to various chemical exposures, infectious diseases have also been identified as causative agents for bladder cancer, including schistosomiasis and bacterial cystitis. Radiation exposure to the pelvis has also been identified as causative for bladder cancer (1,2).

After years of extensive research, much is known about the process of carcinogenesis (4,5). Fundamentally, we now know cancer is due to genetic alterations, usually occurring in somatic cells. It has also become clear that more than one genetic alteration is required for cancer to arise. All of the genetic alterations necessary for cancer development must occur in a single cell. These genetic mistakes become permanent only if they occur during DNA replication. Furthermore, although DNA replication is incredibly precise, spontaneous errors occur each time DNA replicates. Based on these assumptions regarding carcinogenesis, there are ultimately only two ways by which an agent, chemical or otherwise, can increase the risk of cancer: 1) increase the rate of DNA damage directly; or 2) increase the number of DNA replications (4,5). The chemicals which directly damage DNA have been referred to as DNA reactive or genotoxic. I will use the more restrictive term, DNA reactive. Agents which induce cancer by increasing DNA replication are referred to as non-DNA reactive or non-genotoxic, and act by increasing cell proliferation. Most DNA reactive agents also increase cell proliferation at high exposure levels, but usually not at lower exposure levels (6).

An essential aspect of the carcinogenesis paradigm is that all of the necessary errors in the DNA must occur in the stem cell population of a tissue (4). This is the population of cells that provides for replacement and repair of tissues when there is injury or toxicity. Under normal circumstances, when a tissue stem cell divides, it produces a replacement stem cell and a cell that is committed for eventual differentiation. Differentiation is a cell death process. As differentiated cells die, they are replaced by this proliferative process of normal stem cells. When there is toxicity or injury to a tissue, resulting in an overall loss of stem cells in the population, normal stem cells replicate into two stem cells until the stem cell population is replaced, at which time normal differentiation processes can again occur. Every time DNA replication occurs within a normal stem cell, there is a rare probability that a mistake can occur in one of the genes that is necessary for the ultimate development of cancer. As the mistakes that are necessary for cancer accumulate in the cell, during each of the subsequent DNA replications there is a probability of additional mistakes occurring until the ultimate number of mistakes have occurred that lead to the production of a malignant cell. Malignant cells replicate, frequently more rapidly than their normal counterpart, but most malignant stem cells still have the capability of undergoing differentiation, although to a more limited extent than their normal counterpart.

The ''spontaneous'' errors that occur during DNA replication are due to the numerous endogenous chemical alterations that occur on the DNA on a regular basis, including oxidative damage, exocyclic adduct formation, and many other events which occur numerous times daily on the DNA (7). Although most of these chemical alterations of the DNA that occur endogenously are repaired, occasionally one leads to a permanent error in the DNA. DNA reactive carcinogens increase the amount of DNA damage per replication by forming additional adducts on the DNA. Again, although most of these are repaired, some lead to permanent errors in the DNA. By increasing the number of DNA replications, the actual number of mistakes can increase even though the rate per DNA replication does not change.

For chemicals that produce urinary tract cancer, they can either be DNA reactive or non-DNA reactive (8). As indicated above, the first identified bladder carcinogens were the aromatic amines which are DNA reactive. If the bladder carcinogen is not DNA reactive, then it increases bladder cancer risk by increasing cell proliferation. This can be due to direct mitogenesis (one example is known, propoxur in the rat), but more commonly the chemical increases cell proliferation by inducing toxicity with consequent regenerative proliferation. Toxicity can be induced in the urinary bladder either by the formation of urinary solids (precipitate, crystals, or calculi), or the chemical or a metabolite can be cytotoxic directly to the urothelial cells. There is some evidence suggesting that in the animal model extreme abnormalities of urinary composition can lead to toxicity and consequent regeneration, such as extremes in urinary pH or volume, although it is unclear whether this is accompanied by other alterations, including chemical cytotoxicity or formation of solids.

Since toxicity is usually, if not always, a threshold event, chemicals that induce cancer by toxicity and regenerative proliferation consequently have a threshold level (9,10). This is most obvious for agents related to the formation of urinary solids (11,12). Solids will only form if the solubility of the substance is exceeded. This is a physical property of a chemical in the specific solvent milieu, in this case, urine. For such agents, there is a clear threshold response, so that if administration of the chemical is at a dose that is high enough to produce the urinary solids, there is toxicity and ultimately tumor formation. If the dose is insufficient, below the threshold, to produce urinary tract solids, then there is

no toxicity and no tumors are produced. This has been most extensively investigated with respect to substances that produce urinary tract calculi (12). There are numerous such substances, including several which are essential for basic biological processes such as calcium, phosphate, cysteine, glycine, orotic acid, or oxalate (11,13). Numerous agricultural, commercial, and pharmaceutical chemicals also can produce calculi when there are high levels of exposure, such as melamine, terephthalic acid, nitrilotriacetate, sulfonamides, HIV protease inhibitors, and carbonic anhydrase inhibitors. Many of these have been known to produce calculi at high exposure levels, not only in experimental models but also in humans (11–13).

Urinary calculi can form directly from the administered chemical (or a metabolite) or can form from constituents normally present in the urine, such as calcium phosphate, calcium oxalate, or uric acid, secondary to alterations of normal physiological processes resulting in increased concentrations of these substances in the urine (13). Regardless of how the calculus is formed, it acts as an irritant, producing epithelial cytotoxicity, sometimes with full thickness ulceration of the epithelium and consequent hematuria. The amount of toxicity is dependant on numerous variables including the size of the calculi, their number, and the coarseness of the surface.

Sodium saccharin produces bladder tumors in rats when the administration begins at birth and continues for the life of the animal (14). In contrast, administration to mice or monkeys for their lifetime does not produce any effects on the lower urinary tract. In addition to being species specific, tumor formation is also a high dose phenomenon, requiring 25,000 ppm (2.5%) of the diet or higher. The mechanism of action involves dramatic changes in the urinary composition leading to the formation of calcium phosphate-containing precipitate (15). Calcium phosphate precipitate is cytotoxic to epithelial cells, including the urothelium. This leads to a cytotoxic effect on the bladder epithelium with consequent regenerative proliferation and ultimately tumors. Like the situation with calculi, this is a threshold phenomenon based on the solubility of calcium phosphate.

Numerous changes in the urine composition must occur for the calcium phosphate precipitate to form, and if any of these parameters are not affected appropriately, the precipitate does not form. Thus, administering saccharin as the acid rather than as the sodium salt produces an acidic urine which inhibits the formation of the calcium phosphate precipitate (16). Thus, there is no precipitate formation, no toxicity, and ultimately no tumors. In the mouse, the urinary concentration of calcium and phosphate is 10–20 times lower than that of the rat, and is not high enough for precipitate to form, again, a clear indicator of the threshold phenomenon that is involved (15). It turns out that in humans, like monkeys, the urine does not contain adequate amounts of protein nor is the osmolality sufficiently high for the precipitate to occur (15,17). Thus, sodium saccharin is both a species specific and a high dose (threshold) chemical carcinogen.

Inorganic arsenic is also carcinogenic for humans, and the organic arsenical, dimethylarsinic acid (DMA) is carcinogenic in the rat (18). It also illustrates the complex nature of the various phenomena included in the term genotoxicity.

Genotoxicity can involve direct interaction of the chemical with DNA, referred to as DNA reactivity, such as in the case of the aromatic amines (3). Arsenicals do not bind to DNA (19).

Although arsenic is not DNA reactive, numerous *in vitro* studies have demonstrated that it does produce various forms of genotoxicity, resulting primarily from inhibition of DNA repair, oxidative damage, or binding to tubulin, which can indirectly affect DNA (20). Although DNA reactivity can theoretically be non-threshold (see below), these other forms of genotoxicity all involve threshold phenomena (21,22). Thus, it is critical in discussing genotoxicity to specify what type of phenomenon is involved with respect to a given chemical. All except DNA reactivity are clearly threshold phenomena.

In the case of arsenicals, many of the studies showing various types of genotoxicity actually involve concentrations *in vitro* that are higher than concentrations required to kill the cells (18–20). Likewise, *in vivo*, the dose required to demonstrate genotoxicity is usually higher than the dose necessary to produce a proliferative or tumorigenic response. Thus, it is unlikely that arsenicals are carcinogenic by a genotoxic mode of action.

Instead, it is more likely that arsenicals are carcinogenic to the bladder and other tissues by a mode of action involving cytotoxicity and regenerative proliferation (18,23). Arsenicals are metabolized by a sequence of reductions of the pentavalent to trivalent form followed by oxidative methylation of the trivalent species. The pentavalent forms of arsenic are generally quite inactive with respect to toxicity, whereas the trivalent forms are extremely reactive, frequently being lethal to cells *in vitro* at concentrations less than 1 μM. Dimethylarsinous acid (DMA^{III}) and monomethylarsonous acid (MMA^{III}) are particularly cytotoxic to cells. Administration of arsenite, arsenate, or DMA^{V} to rats at high doses produces a high concentration of trivalent arsenicals, particularly DMA^{III}, in the urine which is cytotoxic to the urothelium leading to regenerative hyperplasia. In mice, arsenate and arsenite are able to produce a similar cytotoxicity and regeneration response (23,24) whereas DMA^{V} does not (18), likely be-

cause of its more limited metabolism in the mouse compared to the rat. Similar to other substances which are carcinogenic by a mode of action involving cytotoxicity and regenerative proliferation, it is likely that arsenic carcinogenesis also involves a threshold, despite having some genotoxic but not DNA reactive properties.

DNA reactive carcinogens remain a unique group of chemicals. Their dose response has generated considerable controversy both with respect to genotoxicity and carcinogenicity for several decades. An experiment referred to as the megamouse experiment performed in the 1970's at the National Center for Toxicological Research was designed to try to address this issue (6,25). The reason for it being called the megamouse experiment was that more than 24,000 mice were utilized, with several hundred per dose group so that the level of detection for a carcinogenic response was 1% rather than the usual approximately 10% when the standard 50 or 60 animals are used per group (25). The carcinogen 2-acetylaminofluorene (AAF) was administered in the diet and sacrifices were performed at 18, 24 and 33 months. The doses used in the experiment, 0, 30, 45, 60, 75, 100, and 150 ppm were considerably lower than in previous experiments with AAF.

Quite unexpectedly, the dose response for the two target tissues of AAF in mice, the liver and urinary bladder, was completely different (6,25). The dose response in the liver was nearly linear, whereas the dose response in the bladder had an apparent threshold of approximately 45 to 60 ppm, with a statistically significant incidence detected at 60 ppm but not at 45 ppm. The question remained however, did this represent a true threshold or was the level of detection, even at 1%, inadequate to detect the low incidence of tumors that might occur at the lower doses in the urinary bladder. Based strictly on the dose response curve for tumors, it would appear that this was a threshold phenomenon, since the dose response was similar to that seen with clearly threshold-type carcinogens, such as calculi-forming chemicals, sodium saccharin, or arsenic. Although there is an apparent threshold, this can only be ascertained by evaluating the detailed mechanism that is involved in the carcinogenic response.

Since AAF is a DNA reactive carcinogen, it forms DNA adducts. In an experiment by Beland *et al.* (26), they were able to demonstrate that the dose response for DNA adducts was linear, both for the liver and the urinary bladder, even down to doses that were considerably lower than those used in the megamouse experiment. The DNA adducts were determined at a steady state level after one month of administration.

For the liver, at the concentrations used in this experiment, AAF produces DNA adducts in the normal hepatocytes, but is metabolized to a much lesser extent and forms adducts to a lesser extent in the foci (which represent the intermediate cell population, one step toward the formation of a hepatocellular tumor) (6). At these low concentrations, there is no evident cytotoxicity or other evidence of increased cell proliferation. Thus, the only effect is on DNA damage in the first step in the carcinogenic process, increasing the probability of a critical mistake occurring in the DNA with each replication normally occurring in the hepatocyte population. The background hepatocellular proliferation rate is approximately 50 to 100 days.

In contrast, DNA adducts form in all cells of the bladder since the reactive intermediate is generated in the liver and excreted in the urine (6). Thus, the probability of critical mistakes occurring during DNA replication is increased not only in the normal bladder epithelial cells, but also in cells all along the process in the development of cancer. The number of cells, however, in contrast to the liver, is much fewer, with only approximately 25,000 stem cells in the normal bladder epithelium and several million in the liver. Even with the number of animals in this experiment, the detection rate for increased tumor incidence is still only 1 % above background. At doses of less than 60 ppm in the diet, there are likely to be an insufficient number of tumors generated in this population to develop a statistically significant incidence. However, at doses of 60 ppm, there is an increase in the rate of cell proliferation and an increase in cell number (hyperplasia). This greatly potentiates the effects of the DNA reactivity, since it increases the number of replicating cells that are present as well as having an increase in the probability of critical DNA damage each time the DNA replicates.

Modeling of such processes estimates the tumor incidence to be expected taking into account both DNA adduct formation and DNA replication (6). In normal bladder epithelial cells, the rate of proliferation is extremely low, similar to the liver. At the dose of 60 ppm and above, there is not only an increase in the proliferation rate, but with hyperplasia, there is an increase in the number of cells. Thus, the number of DNA replications is greatly increased. In these modeling efforts, it can be shown that at doses of 60 ppm and above, a statistically significant incidence of tumors above 1% would occur. The apparent threshold can be shown to be not a true threshold, since there are DNA adducts formed and presumably DNA damage at much lower doses. However, utilizing these modeling systems, it can be shown that the expected tumor incidence at doses of 45 ppm and below would yield incidences well below the 1% that is the detection limit for this experiment.

Thus, although the shape of the tumor dose response curve of AAF is similar to that for calculus-forming rodent bladder carcinogens, based on mechanism, one can not conclude definitely that there is a true threshold.

The key events in the process of AAF-induced car-

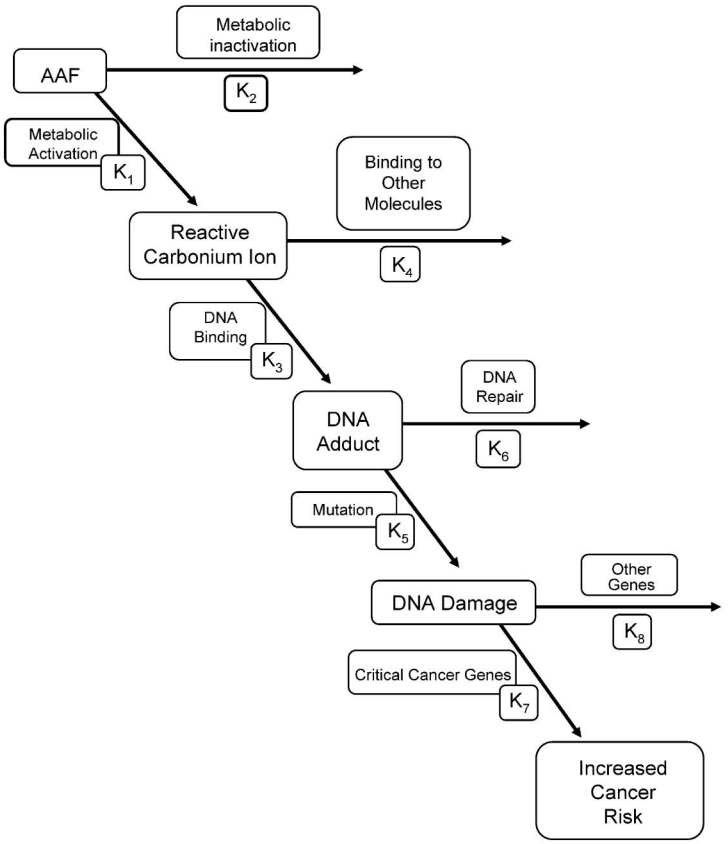

Fig. 1. Competing activating and inactivating processes in AAF carcinogenesis, with imaginary rate constants for each step. Only if all of the activating rate constants (odd numbered constants in the diagram) are zero at some dose can there be a true threshold.

cinogenesis include metabolic activation to a reactive intermediate which leads to the formation of DNA adducts, some of which are not repaired and lead to a mutation in critical cells, ultimately leading to an increased risk of cancer development. However, for each of these key events, there are competing events that would lead to an inactivation of the process (Fig. 1). Thus, not only is there metabolic activation, but there are numerous enzymes involved in metabolic inactivation of AAF. Once the reactive intermediate has formed, it can bind to a variety of chemicals, not only DNA, but protein, RNA, or even water. Binding to any of the substances that are not DNA obviously would not increase the risk of development of cancer. Furthermore, once DNA adducts form, many of them are repaired, again leading to inactivation of the carcinogenic effects of AAF. For those that are not repaired, permanent DNA damage will only occur if the cell is replicating at the time the adduct is present. Furthermore, the DNA adduct could form in one of the genes critical to the development of cancer, or form in one of the many other nucleotides available in the genome. These latter DNA adducts would not increase the risk of cancer development. For cancer to develop, all of the DNA alterations that are required for the development of cancer must occur in a single cell and that cell must be in the stem cell population. Furthermore, there are a variety of cellular repair processes once the critical DNA damage has occurred. These competing events are illustrated in Fig. 1. Imaginary rate constants are given for each of these competing processes, with odd numbered rate constants for the activating processes and even numbered rate constants given for the inactivating processes. It is only when all of the activating rate constants (odd numbered) are zero can a true threshold be possible. It is my impression that one or more of these rate constants at low concentrations can be zero, but we do not yet have the technology with appropriate detection limits to be able to prove the possibility of thresholds for DNA reactive carcinogens.

There are several terms which are used in a confusing fashion and sometimes interchangeably that must be distinguished carefully when addressing the issue of threshold. Most importantly is the issue of threshold vs. level of detection. Also, many individuals have used the terms threshold and non-linearity synonymously, whereas in fact they are distinguishable as illustrated in the example of 2-AAF above. A critical distinction is a true threshold vs. a practical threshold. A true threshold, although likely for DNA reactive carcinogens, has yet to be proven as far as I am aware.

However, we regularly deal with the concept and validity of practical thresholds. For example, numerous natural DNA reactive carcinogens are present in our diet, such as aflatoxin. Because of dramatic developments in technology, we can measure incredibly small amounts of aflatoxin so that it can be detected in virtually all peanut products as well as in many other grain products. Whether or not there is a true threshold in the carcinogenic effects of aflatoxin is unknown. However, a safe level can be estimated for practical purposes so that we can consume peanuts and other products which contain miniscule amounts of the chemical. Such estimates are based on an extrapolation to low doses based on animal and/or human investigations, with an extrapolation estimate of overall risk of 1 in 100,000 or 1 in 1,000,000 individuals. This is then set as a safe level for regulatory purposes, and serves as a practical threshold for carcinogenicity.

In summary, thresholds for genotoxicity are known to occur for genotoxic mechanisms not involving direct DNA reactivity. For DNA reactivity, the issue remains unresolved. Similarly, for carcinogenesis, non-DNA reactive genotoxic and completely non-genotoxic chemical carcinogens clearly have thresholds. For DNA reactive carcinogens, again, this remains unresolved.

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