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THE DECISION-POINT APPROACH FOR SYSTEMATIC CARCINOGEN TESTING*

J. H. WEISBURGER and G. M. WILLIAMS

American Health Foundation, Naylor Dana Institute for Disease Prevention, Valhalla, NY 10595, USA

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Summary—Advances in the understanding of the mechanisms of chemical carcinogenesis suggest new approaches to the practical aspects of the bioassay of carcinogens and regulatory impact, and to the determination of health risk. Chemical carcinogens have been classified into two main types on the basis of their specific properties: (1) genotoxic carcinogens; (2) agents operating by epigenetic mechanisms. Current concepts indicate that genotoxic carcinogens require distinct qualitative and quantitative types of analysis since their fundamental mechanisms of operation are different from those of epigenetic agents. A systematic 'decision-point approach' to carcinogen testing provides for distinction between genotoxic and epigenetic carcinogens. The first set of data points involves the following: (1) structure–activity relationships; (2) mutagenicity assays in prokaryotes; (3) mutagenicity assays in eukaryotes; (4) tests for induction of DNA repair in eukaryotes; (5) tests for sister chromatid exchange; (6) cell transformation. Not all of these have equal sensitivity, specificity and reliability. The sequence of *in vitro* tests permits preliminary decision making. As a second series, limited, relatively rapid, *in vivo* assays involve the following: (1) skin-tumour induction in mice, with and without promotion; (2) lung-tumour induction in mice; (3) breast-cancer induction in rats; (4) identification of early lesions in rodent liver. The data so obtained are considered for decision making and risk analysis. As a last step, a traditional chronic bioassay may be needed only when human exposure to the product is potentially high and/or continuous, or when the above phases of testing have yielded unsatisfactory or, in the case of epigenetic agents, negative results. More research is essential for the delineation of the effects of epigenetic agents, some of which are most important in the aetiology of human cancer.

Introduction

Cancer is a general term for diverse diseases caused by many distinct but specific risk factors, namely different kinds of chemical carcinogens, co-carcinogens or promoters, and also various forms of radiation, and possibly viruses. It is important not only to define the risk factors but also to consider the actual or potential specific target organs in establishing approaches to qualitative and quantitative estimates of risk.

Much progress in understanding some of the actual causes of diverse cancers resulted when complex multifactorial causes were systematically evaluated in relation to a single, specific kind of cancer, such as cancer of the stomach or colon. It was found that the risk factors for diverse cancers were truly distinct (Fraumeni, 1975; Hiatt, Watson & Winsten, 1977; Higginson, 1979; Reddy, Cohen, McCoy, Hill, Weisburger & Wynder, 1980; Wynder & Hoffman, 1979). While chemicals have caused cancer in certain occupations (Saffiotti & Wagoner, 1976; Shubik, 1976), most types of cancer affecting the general public in various parts of the world are due to lifestyle factors that include cigarette smoking, and also specific macro- and micro-nutrients, and nutrition in general (Table 1). Occupational cancer fortunately affects only a small number of individuals, and current scientific and technical advances, especially through the methods discussed in this paper, should enable us to eliminate this kind of risk entirely.

Substantial progress has been made not only towards establishing the risk factors for specific kinds of cancer but in understanding the mechanisms of carcinogenesis (Brookes, 1980; Coon, Conney, Estabrook, Gelboin, Gillette & O'Brien, 1980; Emmelot & Kriek, 1979; Griffin & Shaw, 1979). Study into the nature of the carcinogenic process has permitted the rational classification of chemical carcinogens. The quantitative aspects for each class of chemical carcinogen, however, also need to be considered in more detail than has been done in the past (Weisburger & Fiala, 1981).

This paper presents the evidence for a new view of quantitative and qualitative distinctions in the mode of action associated with diverse chemical carcinogens. These concepts, in turn, bear directly on contemporary requirements for regulatory actions and risk analysis designed to minimize disease, and especially cancer, risks. It will be shown that chemical carcinogens can be divided into two broad categories—genotoxic carcinogens and epigenetic agents. These two categories are further divided into a total of eight subgroups (Table 2).

Risk evaluation for the genotoxic agents, by virtue of their specific mechanisms of action, must necessarily be different from such an evaluation for agents operating by epigenetic pathways. Genotoxic agents interact with DNA and genetic material (Fig. 1). Once cell duplication with the so-generated abnormal DNA has occurred, the reaction is translated into an irreversible alteration in DNA.

*Detailed treatment of this topic can be found in the references listed for Weisburger & Williams (1980) and Williams & Weisburger (1981).

Table 1. *Probable causes of main types of human cancer*

Cause of cancer	Type of cancer	Percentage of total cancer cases in USA	Estimated no. of new cases per year in USA
Occupational	Various	1–5	7850–39,000
Cryptogenic (viruses?)	Lymphomas, leukaemias, sarcomas, (cervix?)	10–15	78,500–118,000
Lifestyle:			
Tobacco related	Lung, pancreas, bladder, kidney	23	181,000
Diet related – nitrite/nitrate, low vitamin C, mycotoxin	Stomach, liver	4	35,000
– high fat, low fibre, broiled or fried foods	Large bowel, pancreas, prostate breast, uterus	43	339,000
Multifactorial			
Tobacco–alcohol	Oral cavity, oesophagus	4	34,000
Tobacco–asbestos, tobacco–mining, tobacco–uranium–radium	Lung, respiratory tract	5	40,000
Iatrogenic			
Radiation, drugs	Various	1	7850

In contrast, the action of agents that operate by epigenetic mechanisms, which are subject to much further research, usually requires their presence at high levels for a long time, and indeed is reversible up to a certain point. So far as is now known, their action is also tissue-specific. For example, bile acids are powerful promoters of colon cancer, but act as inhibitors when tested in the classic mouse-skin system (Reddy *et al.* 1980; Watanabe, Narisawa, Wong & Weisburger, 1978). Saccharin has been the subject

Table 2. *Classes of carcinogenic chemicals*

Type of carcinogenic chemical		Mode of action	Example
Genotoxic:	Direct-acting carcinogen	Electrophile, organic compound, genotoxic, interacts with DNA.	Ethyleneimine
	Procarcinogen	Requires conversion through metabolic activation by host or *in vitro* to direct-acting carcinogen.	Vinyl chloride, benzo[*a*]pyrene, 2-naphthylamine, *N*-nitrosodimethylamine
	Inorganic carcinogen	Not directly genotoxic; leads to changes in DNA by selective alteration in fidelity of DNA replication.	Nickel, chromium
Epigenetic:	Solid-stage carcinogen	Exact mechanism unknown; usually affects only mesenchymal cells and tissues; physical form vital.	Polymer or metal foils; asbestos
	Hormone	Usually not genotoxic; mainly alters endocrine system balance and differentiation; often acts as promoter.	Estradiol, diethylstilboestrol
	Immunosuppresor	Usually not genotoxic; mainly stimulates 'virally induced', transplanted, or metastatic neoplasms.	Azathioprine, antilymphocytic serum
	Co-carcinogen	Not genotoxic or carcinogenic, but enhances effect of direct-acting carcinogen or procarcinogen when given at the same time. May modify conversion of procarcinogen to direct-acting carcinogen.	Phorbol esters, pyrene, catechol, ethanol, *n*-dodecane, sulphur dioxide
	Promoter	Not genotoxic or carcinogenic, but enhances effect of direct-acting carcinogen or procarcinogen when given subsequently.	Phorbol esters, phenol, anthralin, bile acids, tryptophan metabolites, saccharin

of much study in the last 10 years. There is sound evidence that this agent belongs in the category of epigenetic agents and acts as a promoter for cancer of the urinary bladder (Cohen, Arai, Jacobs & Friedell, 1979; Hicks, 1980; Nakanishi, Hagiwara, Shibata, Imaida, Tatematsu & Ito, 1980; Weisburger, 1980). Therefore, attempts to use standard techniques of risk analysis for saccharin have yielded controversial results because such approaches do not apply to this class of agent. In fact, new procedures to define the mode of action of epigenetic carcinogens, and hence risk evaluation, need to be developed.

Thus, current concepts of the mechanisms of carcinogenesis and the above classification of chemical carcinogens have been the basis of a rational sequential system of detecting carcinogens that might present a health risk. We hope that the detection of potential, but as yet unknown, future health risks, as well as the acquisition of knowledge of the health risks associated with and responsible for diverse kinds of existing important human cancers, will be simplified, accelerated and made more economical and reliable by use of such a systematic 'decision-point approach'.

The decision-point approach

The decision-point approach involves five sequential steps in the evaluation of the potential risks associated with the carcinogenicity of chemicals (Table 3). This approach was formulated to incorporate several newer developments in chemical carcinogenesis. Of major relevance was the view that chemicals

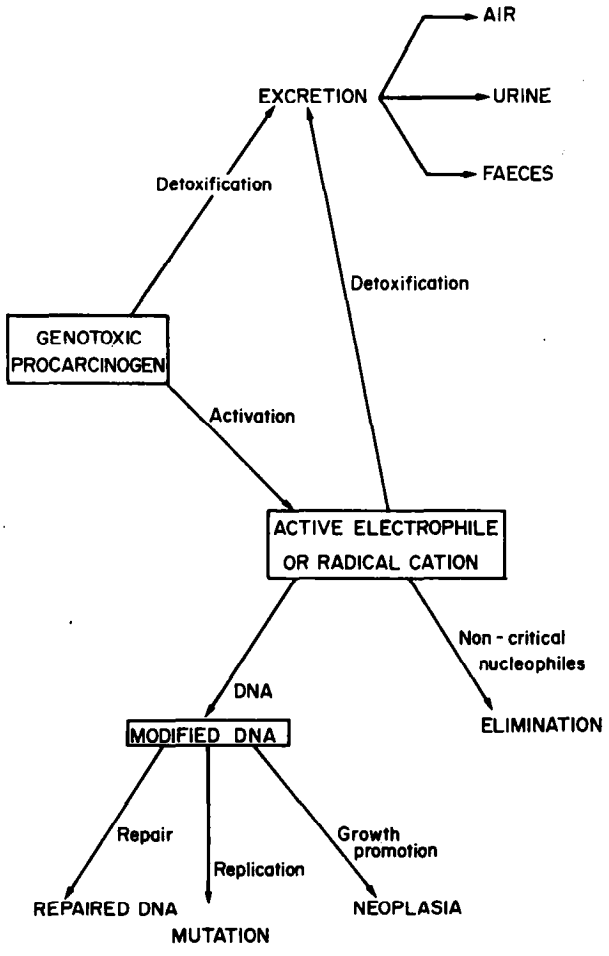

Fig. 1. Schematic presentation of the diverse biochemical reactions involved in the metabolic activation and detoxification of genotoxic carcinogens. For many carcinogens there are reactions leading to detoxified metabolites facilitating excretion. Usually only a small fraction of a dose of a carcinogen is converted, in one or more steps, to more toxic and carcinogenic metabolites, which are reactive electrophiles or radical cations. These metabolites can undergo enzymic detoxification reactions yielding excretable products, or they can react with nucleophiles that are non-critical to the carcinogenic or mutagenic process in competition with the critical nucleophiles, especially DNA. *In vitro* systems have different ratios of activation:detoxification enzymes and distinct levels of non-critical nucleophiles, and thus account for differences found between *in vivo* and *in vitro* assays. The key reaction is with DNA, providing a parallel between mutagenesis and carcinogenesis. This lesion can be repaired, an effect that plays a role in quantitative carcinogenesis and in the specific organ primarily affected by a given carcinogen. Essential elements in the overall process towards mutation or neoplasia are DNA and cell duplication, growth, and promotion. [Based on a figure from Weisburger & Williams (1981).]

Table 3. *Decision-point approach to carcinogen testing*

Stage	Action
A	Consideration of the structure of the chemical
B	Battery of *in vitro* short-term tests
	(1) Bacterial mutagenesis
	(2) Mammalian cell mutagenesis
	(3) Mammalian cell DNA repair
	(4) Mammalian cell chromosome effects
	(5) Mammalian cell transformation
C	Limited *in vivo* bioassays
	(1) Skin-tumour induction in mice
	(2) Pulmonary-tumour induction in mice
	(3) Breast-cancer induction in rats
	(4) Induction of rodent liver altered foci
	(5) Assays for promoters
D	Chronic bioassay
E	Final evaluation

could produce an increase in the tumour incidence in exposed animals, i.e. be carcinogenic, by several distinct mechanisms, each having different theoretical and practical implications. One of these mechanisms, proposed by Drs Elizabeth and James Miller, was through the generation of an electrophilic reactant that could react covalently with cellular macromolecules. Research in a number of laboratories, notably those of the Millers, Brookes and Lawley, Magee and Swann, and Ames (see Brookes, 1980; Grover, 1979; Miller & Miller, 1979; Weisburger & Williams, 1980) has strongly indicated that DNA is in fact the critical cellular target. However, in addition to chemicals that react with DNA, others lacking this property are nevertheless carcinogenic or oncogenic under certain conditions. Among chemicals of the latter type are plastics, hormones, immunosuppressants, cytotoxic agents, co-carcinogens and promoters. Thus, we suggested that chemical carcinogens could be divided into two main categories, on the basis of their capacity to damage DNA (Table 2).

Carcinogens that reacted covalently with DNA were classified as genotoxic, a term first used by Druckrey (see Ehrenberg, Brookes, Druckrey, Lagerlof, Litwin & Williams, 1973), while those lacking this property and probably acting by other mechanisms, were categorized as epigenetic (Weisburger & Williams, 1980). The genotoxic category contains the classic organic carcinogens that damage DNA either through direct chemical reactivity or following metabolism by enzyme systems. In addition, in the light of some evidence for DNA damage or alteration during biosynthesis by inorganic carcinogens, these were tentatively placed in this category.

In contrast, the second category of epigenetic carcinogens is composed of those agents that have not been found to damage DNA, but rather appear to act through other indirect mechanisms. This category contains several classes of agents that operate by distinct non-genotoxic mechanisms, mainly affecting a specific target organ.

Stage A. Structure of the chemical

The first step in evaluating possible carcinogenicity is a consideration of the chemical structure. For specific classes of chemicals such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons, aromatic amines, and aliphatic or cyclic nitrosamines, sufficient experience has accumulated on structure *versus* carcinogenicity to develop preliminary information on potential carcinogenicity on the basis of the structure of the chemical itself and its potential metabolites. There are species-linked differences in metabolism that bear on carcinogenic risks.

Information on structure and metabolism provides a guide to the selection of specific limited bioassays at stage C (below), and as more information becomes available, may also contribute to the selection of relevant short-term tests at stage B.

Stage B. In vitro *short-term tests*

There is general agreement that a battery of such assays is necessary rather than a single test (see Williams, Kroes, Waaijers & van de Poll, 1980). Therefore, the key element in developing a battery of tests is to formulate suitable criteria for selecting the most effective and economical combination of tests. Furthermore, since such testing is becoming quite complex and expensive, it is relevant to attempt to specify the number of tests that are essential without loss of information. The basis for the selection of *in vitro* tests has been reviewed in detail (Williams & Weisburger, 1981).

At this time, a suitable screen necessarily includes microbial mutagenicity tests as developed by Ames, Rosenkranz, Sugimura, Malling, DeSerres, and others. Especially useful are Ames' test involving *Salmonella typhimurium*, and variations thereof (Ames & Haroun, 1980). Mammalian cell mutagenicity tests are included because of the significance of mutagenic effects in the eukaryotic genome, which differs in several important respects from the prokaryotic genome. DNA repair is a specific response to DNA damage, and thus, a test based on this phenomenon provides an endpoint that is highly specific and of great biological significance. A chromosomal test is useful in the battery to delineate chemical effects at a higher level of genetic organization. Cell transformation needs consideration because this test possibly shares mechanisms with those prevailing for *in vivo* carcinogenesis.

In summary, the battery includes tests for (1) bacterial mutagenesis, (2) mammalian mutagenicity, (3) DNA repair, (4) chromosomal effects and (5) cell transformation. All of these tests need elaboration to denote their advantages and especially their limitations.

The results of these tests and structure–activity relationships should be used for preliminary decision making. A positive response in one or more tests indicates suspicion of genotoxicity. This then can be explored further with specifically designed limited *in vivo* bioassays (stage C).

If the *in vitro* test systems yield no evidence of genotoxicity, the priority for further testing depends on two criteria: (1) the structure and known physiological properties (for example, hormonal) of the material; (2) the potential human exposure.

Stage C. Limited in vivo *bioassays*

This stage involves tests that underline available evidence of the hazard of chemicals positive for geno-

toxicity, and yet avoids a long-term chronic bioassay without much loss of convincing data. These tests include: (1) skin-tumour induction in mice, with and without promotion, and with and without initiation, thus giving information on possible initiating or promoting activity of the chemical; (2) pulmonary-tumour induction in mice; (3) breast-cancer induction in female Sprague–Dawley rats; (4) altered foci induction in rodent liver, and (5) variations involving initiators and promoters.

The preceding tests have an advantage in that they truly represent *in vivo* carcinogenesis bioassay. Also, they yield results in less than a year, and in some instances in less than 6 months. Some yield multiple tumours, and thus provide useful semi-quantitative comparative information. Not all classes of carcinogens give positive results in any one of these tests. However, a definite positive result and evidence of genotoxicity from the battery of *in vitro* tests supports the view that a chemical substance or mixture would be carcinogenic. A negative result in any one of the limited bioassays would not necessarily rule out carcinogenic potential. The classes of compounds active in such limited bioassays have been delineated (Williams & Weisburger, 1981).

Stage D. Chronic bioassays

Chronic bioassay is used in the decision-point approach as a last resort for confirming questionable results in the more limited testing, for compounds that are negative in the preceding stages of testing but to which extensive human exposure is likely, or for the acquisition of data on possible carcinogenicity through epigenetic mechanisms. In the latter situation, multi-species and dose-response data are most important, if the results are to be applied meaningfully to risk assessment. Also, if a specific target organ is suspected of being involved, a limited pretreatment with a known genotoxic carcinogen for that organ would facilitate detection and delineation of the effect of the epigenetic agent.

Stage E. Final evaluation

If the decision point approach has led to a chronic bioassay, then fairly definitive data on carcinogenicity would be obtained. However, the results of the *in vitro* short-term tests together with consideration of the structure of the compound must be incorporated into an evaluation of possible mechanisms of action and risk extrapolation to humans. Convincing positive results in the *in vitro* tests coupled with documented *in vivo* carcinogenicity permits classification of the chemical as a genotoxic carcinogen.

If, on the other hand, no convincing evidence for genotoxicity is obtained, but the chemical substance is carcinogenic in certain animal bioassays, then the possibility exists that the chemical is an epigenetic carcinogen. The strength of this conclusion depends upon the structure of the compound and the relevance of the *in vitro* tests.

Quantitative aspects

It is important to realize that health-risk analysis must consider quantitative potential as well as qualitative positive or negative results. It is evident that distinctly different protective measures are needed for the liver carcinogen aflatoxin B_1 (active at 1 ppb*), than for the liver carcinogen safrole (active at 2000 ppm) or acetamide (active at 12,500 ppm). With the powerfully carcinogenic *N*-2-fluorenylacetamide, a lowering of the dose by only one log unit, a factor of 10, converts a very powerful carcinogenic stimulus (200 ppm) to a virtually inactive dose rate (20 ppm). On a larger scale, in the case of cigarette smoke, an individual smoking 40 standard cigarettes per day has a fairly high risk of disease, whereas four cigarettes per day would be a minimal risk. This again is only a factor of ten. Thus, quantitative aspects are most important if the goal of risk elimination, and thus disease prevention, is to be approached in a realistic manner.

Conclusion

The decision-point approach provides a framework, based on current concepts of the mechanisms of carcinogenesis, for the systematic evaluation of the potential mutagenic and carcinogenic hazards of chemicals. This approach can be integrated with other elements in toxicity testing. It is designed to yield a stepwise progression of data acquisition. A carefully conducted evaluation, based on this systematic programme should provide a qualitative and a semi-quantitative sequential data base, and need not necessarily terminate in an expensive and extensive long-term bioassay. This approach provides an effective tool for the protection of the public against environmental carcinogenic and mutagenic factors through health-risk analysis, based on current concepts of risk extrapolation to humans. Convincing positive results in the *in vitro* tests coupled with documented *in vivo* carcinogenicity permits classification of the chemical substance as a genotoxic carcinogen. At that point, an expert group needs to consider whether the value of such a material to human beings at the potential maximal exposure levels is sufficient to tolerate possible human exposure. This risk analysis needs to consider the relative strength of the carcinogen. In the USA, such a value judgment has been made, for example, with respect to the potential risk attached to the powerful carcinogen aflatoxin B_1 as was discussed previously in this paper.

Much more research is necessary on the mode of action of epigenetic agents (see Williams (1981) for a discussion of promoters). Nonetheless, it would appear that risk analysis for such materials will show that the action of such agents will present a definite threshold which can be determined experimentally. Thus, the hazards of human exposure may be mainly of a quantitative nature. Furthermore, in evaluating the effects of intermittent use or exposure to such agents it should be borne in mind that their action is reversible up to a point.

In summary, the proposed testing approach, which embodies new concepts in chemical carcinogenesis, provides for systematic yet economical testing, the results of which can be applied to flexible, rational health-risk analysis.

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*b = 10^9.

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