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ABILITY OF SHORT-TERM TESTS TO PREDICT CARCINOGENESIS IN RODENTS*

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INTRODUCTION

Carcinogenesis is a complex, multistage process. Although an initial step in the process is believed to be the result of a genotoxic or mutagenic event, later stages are poorly understood. One of the major impediments to the study of carcinogenesis, and our ability to identify agents with tumorigenic potential, is the fact that the process is often protracted; the development of a chemically induced tumor may encompass the majority of the expected lifetime of the animal. This has given impetus to the development of short-term tests (STT) that can predict the carcinogenic potential of a chemical or serve as a model for one of the steps in the process.

Numerous STT for genotoxicity have been proposed and developed, but attempts to evaluate these assays have suffered from the inadequate number of known noncarcinogens tested (1, 2). By having results on comparable numbers of carcinogens and noncarcinogens, not only can the sensitivity (i.e. the proportion of carcinogens correctly identified) of each assay, or combination of assays be estimated, but also the specificity (i.e. the proportion of noncarcinogens correctly identified) and the concordance (i.e. the proportion of

carcinogens and noncarcinogens correctly identified). Also, the estimation of predictivity, (i.e. the proportion of carcinogens among chemicals producing a positive response), which is highly dependent on the prevalence (i.e. the proportion of carcinogens among the chemicals tested), depends on an adequate number of tested noncarcinogens.

Although the agreement among in vitro genotoxicity assays appears to be quite good (3), it is not perfect—nor in the real world would one expect it to be. As more chemicals are tested in multiple assays, discrepancies among test results on individual chemicals will increase. If complete agreement is required among assay results, these discrepancies will present a problem in categorizing a chemical as genotoxic or, especially, as nongenotoxic. Possible solutions to this problem are to use an algorithm for "consensus" among the assays or to choose one or a few assays as primary indicators. Given the high degree of agreement among four in vitro assays examined (see In Vitro Genotoxicity Assays), the National Toxicology Program (NTP) has chosen the latter approach, using mutagenicity in *Salmonella typhimurium* as the primary in vitro assay for genotoxic chemicals.

In an effort to understand the relationship among chemical structure, mutagenicity, and carcinogenicity, Ashby and coworkers (4, 5) evaluated data on 264 chemicals. They concluded that: (*a*) there is a 90% correlation between a structural alert (usually an electrophilic group) on the chemical and mutagenicity in Salmonella, (*b*) a mutagenic response in Salmonella was predictive of carcinogenicity in rodents, and (*c*) approximately one third of the rodent carcinogens lack an electrophilic group and are not mutagenic in Salmonella. The electrophilic nature of the chemicals bearing structural alerts suggests that these chemicals, or their metabolites, may interact directly with DNA, and the Salmonella results for these chemicals show their genotoxic potential. Chemicals that do not fit these criteria: (*a*) may be nongenotoxic; (*b*) may have genotoxic potential via one or more indirect means, e.g. production of oxygen radicals; or (*c*) may exhibit genotoxic potential in vivo, though not in vitro.

Two approaches are being used to complement in vitro genotoxicity assays in the identification of potential carcinogens. The first is to evaluate genotoxic activity in vivo; the other approach is to identify experimental endpoints, other than genotoxicity, that are predictive of carcinogenicity. The former is the easier of the two because many of the in vivo assays are modifications of existing in vitro genotoxicity assays.

IN VITRO GENOTOXICITY ASSAYS

Development of Chemical Mutagenesis Testing

Attempts to identify human carcinogens by STT began with the use of a bacterial mutagenesis assay. The first screening of chemicals was reported by

Hemmerly & Demerec (6) who studied mutagenicity in *Escherichia coli* of "chemicals . . . of interest in cancer research." Iyer & Szybalski (7), who developed the spot test method and used it to test a number of chemicals for mutagenicity in *E. coli,* commented on the "close association between the carcinogenic effect in mammals [of chemicals] and the mutagenic effect on bacteria" that was found in the earlier work of Demerec.

The first large scale use of a bacterial mutagenicity test to screen chemicals was, ironically, to identify potential antitumor agents (8). There was a presumption that mutagenicity would be an effective biological correlate with antitumor activity. In a survey of 431 substances that included 62 antineoplastic agents, the known antineoplastic agents comprised 86% of the strong mutagens and 45% of the weak mutagens. Interestingly, 43% (9/21) of the organic chemicals identified as human carcinogens were originally prepared and administered to humans as antitumor agents or as components of antitumor therapy (9).

The early chemical mutagenicity tests were performed prior to the recognition that many chemials require metabolic activation to be mutagenic, and as a result relatively few mutagens were identified. It was not until the early 1970s that Malling (10) demonstrated the effectiveness of rodent liver preparations in metabolizing chemicals to bacterial mutagens in vitro. Ames and his coworkers (11–13) used this technique to show that chemical carcinogenicity and bacterial mutagenicity were closely associated and that many rodent carcinogens were bacterial mutagens. Since that demonstration of the agreement between carcinogenicity and mutagenicity more than 100 STT have been proposed for carcinogen identification (14, 15). These tests encompass a variety of genetic endpoints and range across the phylogenetic scale from viruses to whole mammals. At the same time, a number of metabolic activation systems were developed because the microbial and cultured mammalian cells used to detect genetic damage do not possess the metabolic capabilities of whole animals.

The major impetus for using in vitro STT to identify carcinogens and noncarcinogens was the seminal publication of McCann et al (13) who showed that the Salmonella mutation assay exhibited high sensitivity and specificity; approximately 90% of the carcinogens and noncarcinogens were correctly identified using Salmonella. Publications from other laboratories (Table 1) were largely in agreement and provided further impetus to the incorporation of Salmonella into ongoing and proposed carcinogen identification programs. At the same time, similar high values were imputed to a number of other STT, although supporting data were usually not available.

Recent studies with Salmonella, however, have yielded lower sensitivities and specificities. These studies were performed using chemicals tested for carcinogenicity by the National Cancer Institute (NCI) and the NTP using a

Table 1 Carcinogen and noncarcinogen identification by *Salmonella typhimurium*

Reference	Chemicals	Prevalence	Sensitivity	Specificity	Predictivity	Concordance
13	239	.74	.88	.85	.95	.87
121	139	.70	.93	.74	.89	.87
122	53	.70	.68	.88	.93	.74
123	120	.48	.91	.94	.93	.93
124	271	1.00	.77	-[c]	-	-
125	89	.78	.64	.75	.90	.66
126	89	.92	.76	.57	.95	.74
127[a]	87	.57	.44	.70	.67	.55
85[a]	60	.55	.76	.59	.69	.68
128[b]	160	.96	.80	.67	.98	.79
34[a]	73	.60	.48	.86	.83	.62
129[a]	224	.55	.54	.70	.69	.61
2[b]	270	.83	.78	.62	.91	.76
3[a]	41	.56	.52	1.00	1.00	.73
	(114	.59	.48	.91	.89	.66)[d]

[a] NTP database
[b] EPA Gene-Tox database
[c] Could not be calculated
[d] Combined dataset from (3, 34)

protocol originally defined by the NCI (16, 17). In general, the classes of chemicals examined in these studies are different from the chemicals examined in prior studies. The earlier carcinogen database was composed principally of polycyclic aromatic hydrocarbons, aromatic amines, nitrosamines, and alkylating agents, whereas the NCI/NTP database had relatively few chemicals of these classes and high proportions of chlorinated hydrocarbons, hormonally active chemicals and phthalates. Based on an evaluation of 114 of the most recent chemicals to be tested for carcinogenicity by the NTP (5), only 48% of the carcinogens were identified by the Salmonella assay. However, a positive response in Salmonella had a predictivity of 89% (3).

As was mentioned above, numerous STT have been proposed for identifying carcinogens. The most commonly used in vitro mutagenicity tests have been those that measure gene mutation in bacteria, and gene mutation and chromosome damage in cultured mammalian cells. For a more complete listing of STT, the reader is referred to Hollstein et al (14) and IARC (15).

The most widely used test is the *S. typhimurium*/mammalian microsome assay (Ames test) developed by Ames (11, 12, 18). In this test, a series of histidine-requiring mutants is used, each sensitive to reversion by a different type of mutational event. The Salmonella tester bacteria are mixed with the test chemical and a mammalian liver extract containing the cytochrome P450 monoxygenase system (for exogenous metabolic activation) and plated onto

minimal agar. If mutation is induced, it is evidenced by colonies of cells that are histidine independent, and therefore can grow on the minimal medium. The histidine-requiring parental cells cannot grow into colonies on minimal medium.

The cultured mammalian cell assay systems most widely used for mutagenicity testing use as targets the *tk* gene in mouse lymphoma L5178Y cells (19, 20), and the *hgprt* gene in Chinese hamster ovary or Chinese hamster lung (V79) cells (21, 22). In the L5178Y system, the mutated cells are identified by the loss of their ability to synthesize thymidine kinase, and the resulting resistance to trifluorothymidine or other antimetabolites. The Chinese hamster cell systems measure the loss of the enzyme hypoxanthine-guaninephosphoribosyltransferase (HGPRT), resulting in the ability to survive and form colonies in the presence of the metabolic inhibitor, 6-thioguanine. Similar to the bacterial assays, the tests are performed in the presence and absence of a liver homogenate metabolic activation system.

Chemically induced chromosome breakage is generally measured cytologically as the induction of chromosome aberrations (ABS), which are scored as breaks or rearrangements that are visible in cells at metaphase (23, 24). Another endpoint is the induction of sister chromatid exchanges (SCE) that involve the reciprocal exchange of DNA duplexes between sister chromatids of the same chromosome (25). These are visualized using special chromosome staining techniques. Although SCE occur in untreated cells and are not known to be associated with any adverse effects, they are induced by known genotoxic agents and offer a relatively sensitive and reproducible method of detecting the chromosomal effects of test chemicals. Both the ABS and SCE tests are performed in the presence and absence of metabolic activation and can be carried out in most mammalian and human cell types.

Testing Schemes

The concept of a 'test battery' for detecting mutagens was first proposed in the Mrak Commission report (26), which recommended the use of three specific in vivo rodent tests and "ancillary microbial systems." This report was addressed primarily to the testing of pesticides for their ability to cause heritable genetic damage. Subsequent proposals addressed the 'Tier' system, in which different tests were used at different stages in testing, depending on the responses obtained in the earlier stages (27, 28), and the 'Battery' in which a number of tests were applied contemporaneously (29–33). Subsequent proposed batteries adopted the same testing philosophy but varied the tests to be used. Some of these test batteries were designed to identify mutagens out of concerns for human genetic damage, and others out of concerns for carcinogenicity. However, all these approaches assembled tests that measured different genetic endpoints. Tests for gene mutations in bacteria

and chromosome aberrations were considered the primary tests, although other chromosome or DNA-damage assays were also included. Assay(s) for gene mutation in mammalian cells were always included for confirmation of the bacterial responses.

One premise behind the use of a test battery is that some carcinogens will cause only point mutations, others will cause only chromosome aberrations or other chromosome damage, and yet others will produce only numerical chromosome abnormalities (aneuploidy). Another premise is that the tests will complement each other, so that a carcinogen 'missed' by one STT would be detected by another. Finally, in both the tier and battery approaches, it is assumed that a positive at a higher tier, or a higher level test, will strengthen the significance of the positive result obtained in the lower level test. The validity of these premises was first tested by Tennant et al (34), using 73 chemicals, and Zeiger et al (3), using an additional 41; they showed that there was no complementation among four widely used in vitro STT (Salmonella mutagenesis, L5178Y cell mutagenesis, ABS, and SCE in Chinese hamster ovary cells). That is, if a carcinogen was not identified by one STT, none of the other STT preferentially detected it. The other assays all detected higher proportions of carcinogens than did Salmonella, but had lower specificities. Overall, Salmonella correctly identified carcinogens and noncarcinogens 66% of the time, compared with 59–61% for the other three tests. There was also a high degree of agreement among the tests; the carcinogens that were genotoxic were generally positive in more than one test. These results call into question the concept of a battery of STT as it applies to the detection of genotoxic carcinogens.

These and other studies during the past few years showed that a high proportion of rodent carcinogens are not detected as mutagens by the commonly used in vitro STT (34, 3), and that some in vitro tests detect as positive a relatively high proportion of noncarcinogens (1, 35).

IN VIVO GENOTOXICITY ASSAYS

The use of in vivo STT has increased substantially as the limitations intrinsic to in vitro STT have become increasingly apparent (3, 34, 36). It is becoming clear that the mutagenicity of most chemicals can be detected by the use of only one or two in vitro assays and that in vivo tests, particularly those for cytogenetic endpoints, may have a critical role both in determining the in vivo activity of mutagens identified in vitro and in detecting a unique group of mutagenic carcinogens that are not detected in vitro (37).

A wide variety of STT are available for assessing the genetic effects of chemical agents in the somatic tissues of whole animals (see 38). These in vivo STT are generally conducted to predict potential carcinogenicity via

somatic cell mutation, or germ cell mutagenicity of chemicals. In vivo somatic cell tests can be divided into three general groups based on the tissue or cell types in which effects are determined. The first group makes use of a tissue because it can conveniently provide information on the genotoxic effects of a test chemical, not necessarily because it is the tissue of primary concern. The most commonly used tissue is the bone marrow and the most commonly employed endpoints involve effects on chromosomes (39), e.g. ABS, micronuclei (MN), and SCE. The bone marrow is used for these studies because of the ease of obtaining a large number of mitotically active cells.

The assays for SCE and ABS are not discussed in detail in this review; descriptions can be found in Latt et al (25) and Preston et al (40), respectively. Chromosomal aberration tests are the best established assays for in vivo genetic toxicity in rodents. The most thorough and objective assessment of the performance of the in vivo ABS and SCE tests will occur when testing is completed on the 73 chemicals previously used to assess the performance of four in vitro STT (34). Although ABS are known to be associated with a variety of adverse health effects, performance of the assay is demanding in both time and expertise. For this reason, tests for MN (41) have recently become more widely used and are considered by many to be a suitable replacement for chromosomal aberration tests as initial screens for in vivo genetic toxicity.

Micronuclei are DNA-containing, membrane-bound, cellular inclusions that result from the exclusion of chromosomes or chromosome fragments from the daughter nuclei following nuclear division in dividing cells. This anomaly in nuclear division results from the displacement of chromosomes or fragments from the spindle apparatus. Thus, chemically induced increases in MN are a measure of induced chromosomal damage (41, 42). They are visualized using DNA stains and can be scored in nucleated cells, but are more easily detected in anucleate cells such as erythrocytes. Whole chromosome loss may be distinguished from chromosome breakage by the detection of kinetochores in the MN (43). The MN test is applicable to a range of tissues, but is most often conducted in polychromatic erythrocytes from the bone marrow. In addition to the ease of scoring and objectivity offered by MN, they can be scored in the peripheral blood of mice and thus are fully compatible adjuncts to other mouse toxicity studies such as 14 or 90 toxicity tests. Approximately 50 of the 73 chemicals used to examine the in vitro STT are being evaluated in the mouse bone marrow MN assay in an effort to characterize its performance.

In the second group of in vivo somatic cell tests are assays that have been devised to look directly at the tissue of concern. Because the liver is an organ in which chemically induced tumors often occur in rodents, test systems have been developed to assess the genotoxic effects of test chemicals in hepato-

cytes (44, 45). The most widely used is a test for unscheduled DNA synthesis, a measure of repair of chemically induced DNA damage. In this test, tritiated thymidine is used to monitor repair synthesis in cells that normally do not synthesize DNA. Incorporation of the radioactivity is determined by either scintillation counting or autoradiography. Similar techniques have been applied to other tissues such as kidney and bladder epithelium (46).

In the third group are methods that allow the assessment of DNA damage in a wide range of tissues. With these systems one could screen several tissues for genotoxic effects or could look for genetic damage in selected organs or tissues that are known or suspected to be affected by toxicity. These methods are in various stages of development. One method is the detection of DNA adducts using ^{32}P postlabelling (47) or immunological techniques (48), and a second utilizes transgenic animals. Adduct detection assays are already being used widely. Although the assay does not detect mutation, the presence of adducts or altered nucleotides is a clear demonstration of interaction between the test chemical and DNA.

Transgenic mouse systems are being developed in which selected genes that will serve as the target for mutagenesis are engineered into the mouse genome (49, 50; J. M. Short, personal communication). Because these target genes are present in every cell, virtually any tissue may be evaluated for evidence of induced mutation. These assays are expected to yield invaluable information on the nature of chemically induced mutations in mammalian somatic cells and on the relationship between genetic damage and induced tumors. Mice bearing an activated cellular oncogene as a transgene exhibit an increased incidence of tumor formation in many of the tissues expressing the transgene (51–53), and may be sensitized to the carcinogenic potential of some chemicals.

Test Design

Factors that must be considered in the selection and design of in vivo tests include the species and sex of the animal to be used, route of exposure, exposure levels, and the number and schedule of exposures to be administered. General agreement has not been reached on these factors. For the NTP-testing activities, choices have been made in these matters based on consideration of the purpose for which the tests are being conducted, and a compromise made between the cost and information to be obtained.

In vivo cytogenetic studies are generally conducted in male B6C3F1 mice. The mouse was chosen because of extensive experience with this rodent. The B6C3F1 hybrid was selected specifically because of its use in NTP long-term toxicity and carcinogenicity studies. The male is used because of its more stable physiology (lack of estrous cycle) and because no chemical has been shown to induce aberrations in one sex but not the other.

If it is deemed important to conduct tests in two experimental groups, it may be better to use one sex of two species rather than two sexes of one species. This would provide for data from genetically diverse groups and may offer the chance to detect effects that are species-specific. Such an approach is supported by the work of Albanese et al (54) who reported the results of bone marrow micronucleus tests in rats and mice using 1,2-dibromo-3-chloropropane and 1,2-dimethylhydrazine. The former was positive only in rats while the latter was positive only in mice.

Another major factor to be considered in designing an in vivo genetic STT is the level of chemical exposure. This includes the number of doses, the dose levels, and the number of exposures. Typically, in vivo tests have been conducted using at least three dose levels, the top dose approximating the highest dose tolerated and the other two being fractions of this. A single exposure to the selected doses is the most frequently used treatment regimen. Because chemicals differ in the rates at which they reach the target cells, and because they may only affect cells in particular stages of the cell cycle, it is often necessary to harvest cells at multiple times following treatment. This must be done to assure that, if an effect is induced, the endpoint is scored at or near its transient peak.

Three dose levels, as described above, remain an adequate basis for an in vivo test, but recent proposals to use multiple exposures offer the advantages of reducing the number of animals and eliminating the necessity for multiple sampling times (55, 56). This multiple exposure treatment regimen has been most frequently used in the bone marrow MN test (42). The studies of Tice & Erexson (56), and interlaboratory collaborations being conducted in Japan, the UK, and the US, to evaluate the utility of a multiple exposure MN protocol, may soon resolve some of the questions about the most appropriate treatment regimen for this test.

A test design question that is less likely to be resolved in the near future regards the route of administration of the test chemical. The two most frequently used routes in short term rodent studies are intraperitoneal injection and gavage. The pros and cons of these two routes were recently discussed in the literature (57, 58); no clear consensus has yet developed. The facts remain that, in general, intraperitoneal exposures lead to higher systemic exposures and gavage leads to a more "natural" exposure, along with more normal absorption, metabolism, and distribution. The choice between these two routes should be influenced by the purpose of the test to be conducted, and will certainly be affected by the testing philosophy of the person or organization conducting the test.

Within the NTP's genetic toxicology program, efforts are underway to integrate in vivo genetic toxicity tests into the animal toxicity studies that precede and are used to design the long-term toxicity and carcinogenicity studies. Not only does this approach help minimize the number of animals

used, it provides genetic toxicity results from exposures that are considered relevant to humans, i.e. feed, water, inhalation.

ASSAYS FOR NONGENOTOXIC CARCINOGENS

The multistage model of carcinogenesis proposes that there are progressive stages in the development of a tumor. The steps in the process of carcinogenesis are poorly understood. An early step, initiation, is believed to depend on the occurrence of a mutation. Other steps or alternate pathways in tumor development may not depend on mutagenesis because a significant proportion of carcinogens identified by the NCI/NTP have not exhibited genotoxic potential (34). A number of biological processes have been suggested as relevant to carcinogenesis; and experimental test systems are being designed around these processes. Several systems having endpoints that do not depend on mutation are described below.

Tumor Promotion

Because carcinogenesis is a complex multistage process, carcinogens, including those that act by nongenotoxic mechanisms, may influence the process in diverse ways. For this discussion, the term tumor promoter is used to describe chemicals that increase the frequency of neoplasms in vivo after a treatment with a noncarcinogenic dose of an initiating chemical. The resulting neoplasms may regress following the termination of promoter treatment, but with continuing treatment a small percentage will progress to become malignant (59). It is important to realize that initiation or promotion activity may be only one characteristic of a chemical and that many carcinogens may possess all activities needed to give a tumor. Several assays have been developed to study the activity of promoters and may be used to identify chemicals with promoter activity. These include assays to follow the development of tumors in the mouse lung and skin, and the rat liver, mammary gland, bladder, kidney, colon, and thyroid (60). The two most extensively studied systems are the mouse skin assay (61, 62) and the rat liver focus assay (63, 64).

The mouse skin system has been used to study the greatest number of chemicals for promoting activity (61). Although many strains have been used in this assay, SENCAR is currently widely used because of its increased sensitivity to promotion (62). In this procedure, the skin on the back is first treated with a noncarcinogenic dose of an initiating chemical followed by repeated treatment with a potential promoter, and the appearance of dermal papillomas is scored visually. The assay generally requires about one year to complete. The papilloma incidence is linearly related to both the dose and frequency of promoter application (65).

The rat liver focus assay has also received considerable attention as a

multistage carcinogenesis model (66, 67). In this assay, a rat is given a partial hepatectomy, followed by treatment with the initiating agent and then feeding or injection of the potential promoter for six months or longer. Hepatic foci are scored histochemically on the basis of an altered enzyme activity, such as gamma glutamyl transpeptidase, ATPase, or glucose-6-phosphatase. Recent studies have attempted to quantitate the relative initiating and promoting activity of various hepatocarcinogenic agents (64). Dose responses could be obtained with both initiating and promoting agents. The potency of promoters ranged over 8 orders of magnitude, while initiators showed a narrower range of potency.

Mammalian Cell Transformation

Transformation in eukaryotic cells is the conversion to a state of unregulated growth in vitro. Because the morphology of the cells and their growth behavior resemble the growth of tumor cells, transformation of mammalian cells by chemicals has been proposed as a biologically relevant endpoint for identifying potential carcinogens. Efforts by several investigators (68–71) have demonstrated the capacity of a variety of chemicals to enhance the transformation of cells. Although many of the chemicals that were examined are genotoxic, transformation is not necessarily a genetic event and may not depend on mutagenesis. Transformation has been demonstrated when cells are treated with a nontransforming dose of an initiating chemical followed by continuous treatment with a known promoter (72–75). Transformation induced by tumor promoters is dependent on the dose and frequency of treatment and is generally reversible on the cessation of treatment.

Dependence upon altered morphological and other phenotypic changes to indicate transformation has imposed subjectivity on efforts to quantitate the assay results; and the instability of the neoplastic phenotype has hindered the verification of the results. For these reasons, the development of transformation assays for chemical screening has lagged behind that of other assays discussed here.

Chemically induced transformation of normal cells in vitro to an altered morphological phenotype was first demonstrated in primary Syrian hamster embryo (SHE) cells by Berwald & Sachs (76). Primary embryonic cell cultures possess a normal diploid karyotype, exhibit a greater endogenous metabolic capability than established cell lines, and have a low background incidence of spontaneous transformation. Morphological transformation of SHE cells is characterized by colonies possessing altered growth patterns, generally a piling up of cells in the center of the colony and disoriented growth around the periphery. Because the scoring of this phenotype is subjective, it is important to demonstrate quantitative and qualitative reproducibility among laboratories. Three laboratories (77) were able to

obtain a qualitative consensus on the activity of eight model chemicals. Subsequently, LeBouef et al (78) reported a significant improvement in clonal proliferation of SHE cells. Under their conditions of lower pH, the transformation frequency induced by several model carcinogens was significantly increased, and the morphology of the transformed foci became more clearly defined.

An assay system that measures the ability of chemicals to enhance Simian adenovirus (SA7) transformation of SHE cells was developed by Casto (79). The adenovirus has the capacity to transform hamster, rat, or human cells and the enhancement assay apparently reflects the capability of the chemical to increase virus integration (80). Transformation is scored as the ability of colonies to grow in soft agar. This assay has been used to test a variety of chemicals in an interlaboratory comparison (81) which demonstrated that consistent results could be produced in the SHE/SA7 assay using structurally diverse coded chemicals.

Two mouse cell lines, Balb/c 3T3 and C3H/10T½, have been developed for chemical transformation studies (82, 83). Established mouse cell lines have been chosen for chemical transformation assays because they exhibit a discrete pattern of induced morphologically altered colonies, and the relatively greater efficiency with which they can be transformed by certain chemicals. However, the assays suffer from the fact that the cells are karyotypically abnormal, are immortalized, have reduced endogenous metabolic capability compared to that observed in primary cell cultures (84), and have a relatively low sensitivity for detecting a diverse group of carcinogens (85).

Other Endpoints

In addition to the transformation assays, several in vitro cell systems for use in the detection and study of nongenotoxic carcinogens are at various stages of development. Hepatic cell proliferation, metabolic cooperation, and induction of protein kinase C (PKC) are discussed here because they have received the greatest amount of study. However, at this time these systems have not been well validated and may be more useful for mechanistic studies than as predictors of carcinogenesis. The induction of aneuploidy has been suggested as a contributing factor in tumorigenesis (86). A number of in vitro and in vivo assays are under development or are being evaluated. Other approaches to the study of nongenotoxic carcinogens have also received some attention, but are at an even more rudimentary stage of development. These include altered or blocked cellular differentiation (87, 88), oxygen radical production (89–92), gene amplification (93), and altered DNA methylation (94).

HEPATIC CELL PROLIFERATION In the mouse skin and rat liver tumor promotion assays, cell proliferation as a result of toxicity or mitogenic stimuli

is often associated with promotion. Because of this observation, an in vivo/in vitro approach to measure scheduled DNA synthesis (SDS) in cultured hepatocytes after treatment in vivo with the test chemical has been developed (95). Both genotoxic and nongenotoxic carcinogens can induce SDS; however, the observation of chemically induced SDS in light of other biological effects, such as genotoxicity and hepatotoxicity, may be useful in determining the biological effects of a potential carcinogen. For example, methapyriline induces SDS and appears to be hepatotoxic, but not genotoxic in the liver (96), whereas di(2-ethylhexyl) phthalate induces SDS, apparently without genotoxicity or hepatotoxicity (97).

METABOLIC COOPERATION Of the STT proposed for nongenotoxic carcinogens, the V79 metabolic cooperation assay (98) has been the most widely applied (99). The biological basis of this assay is that cells communicate via gap junctions (100). Inhibition of this communication is believed a contributing factor in the process of carcinogenesis by allowing an initiated cell to overcome growth control mechanisms imposed by the surrounding cells (101, 102). In a three laboratory study using 23 coded chemicals, the sensitivity in detecting tumor promoters was estimated to be about 60% (103). The assay is currently being evaluated using the 73 chemicals reported by Tennant et al (34). The metabolic cooperation assay has been extended to other cell types, including liver cells (104, 105).

ACTIVATION OF PROTEIN KINASE C PKC is a Ca^{2+}/phospholipid-dependent protein kinase that phosphorylates several intracellular proteins, including epidermal growth factor receptor (106), $pp60^{src}$ (107), p21 *ras* (108), and others (109). PKC is the predominant cellular receptor for tumor-promoting phorbol esters and is activated by these compounds (110, 111). Other classes of tumor promoters, such as indole alkaloids and polyacetates, also activate PKC (112). In addition, the production of oxygen radicals may be mediated by membrane-bound PKC in response to activation by phorbol 12-myristate-13-acetate (113). Over-expression of the PKC gene in transfected cells leads to altered cell-growth characteristics, such as reduced dependence on serum, higher saturation densities, anchorage independence and enhanced tumorigenicity when implanted into nude mice (114, 115).

ANEUPLOIDY It has been suggested that events giving rise to aneuploidy, an abnormal chromosome number, may be mechanistically involved in cellular transformation in vitro and in tumor formation in vivo (for review see 86). Recent evidence (116) suggests that aneuploidy in the mouse skin may be related to the conversion of benign papillomas to malignant carcinomas. Several assays have been or are being developed to test chemicals for the

potential to induce aneuploidy (117). These range from the in vitro study of microtubule assembly through fungal and cultured mammalian cell systems to the genetic analysis of the progeny of chemically exposed individuals. Only a few chemicals have been examined in more than one of these assays (118). While the fungal assays are the most highly developed, there is some question as to the ability to extrapolate to mammals because of the inability of fungal microtubules to bind colchicine (119). Among the mammalian assays for aneuploidy, the MN assay accompanied by staining for kinetochores is becoming widely applied (43).

In most of the assays for nongenotoxic carcinogens too few chemicals have been studied to estimate the predictive value of the assay. The effect of nongenotoxic carcinogens on cellular events such as gene amplification and regulation, chromosome segregation, oxygen radical production, intercellular communication and intracellular signal transduction needs further investigation. Once the relation of these events to the carcinogenesis process is understood, it may be possible to construct a battery of assays, each measuring a different event, to identify the biological effects of exposure to specific nongenotoxic carcinogens.

FUTURE DIRECTIONS

The field of genetic toxicology has seen a great increase in the number of genotoxicity assays that have been developed to identify potential carcinogens. Only recently have those assays come under close scrutiny for their utility in this endeavor. The in vitro genotoxicity assays agree well with each other (3, 34), suggesting that they in fact identify the same or similar types of damage with varying degrees of sensitivity. This in itself is reassuring. It is also reassuring that predictivity may be quite high (Table 1). These two observations suggest that the use of one or a few representative in vitro genotoxicity assays may be sufficient to identify the majority of the mutagenic carcinogens.

The in vitro genotoxicity assays, however, miss a fairly high proportion of carcinogens, including some that are very potent (34). This is not surprising, perhaps, given our current understanding of carcinogenesis as a complex process in which mutagenesis plays a role in some, but not all, of the steps. Two potential problems arise from the exclusive use of in vitro genotoxicity assays to predict potential carcinogens. The first is that promutagens may not be activated by the artificial metabolic activation used in the in vitro assays. A possible indication that this might be a serious problem is that two carcinogens that were not found to be mutagenic in the Salmonella assay produced liver tumors with novel mutations in the *ras* oncogene (120). Although a causal relationship has not been proven between the oncogene mutations and

the tumors recovered, cytotoxic lesions were not found in the livers of the treated animals, and the spectrum of mutations in the induced tumors was significantly different from that found in the spontaneously arising tumors. An approach to this problem is to develop an in vivo assay that will allow mutations to be scored in any of a variety of tissues, preferably the same tissues as are examined in carcinogenesis assays. Such assays utilizing transgenic mice are currently being developed (49, 50).

The second problem, as mentioned above, is the existence of nongenotoxic carcinogens. Short-term assays are being developed to simulate suspected events in the carcinogenesis process. Given that a number of physiologically discrete events may be involved in the development and progression of a tumor, a battery of tests, each simulating a relevant event, may be needed. The development and use of these assays will provide an opportunity to fill in the many gaps in our understanding of chemically induced carcinogenesis and will substantially improve our ability to predict potential chemical carcinogens.

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