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The Use of Short- and Medium-term Tests for Carcinogens and Data on Genetic Effects in Carcinogenic Hazard Evaluation

Edited by D.B. McGregor, J.M. Rice and S. Venitt

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CONSENSUS REPORT

Introduction

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme to evaluate the carcinogenic hazard of chemicals to humans and to produce monographs that review and summarize the published, openly available data relevant for this purpose on individual chemicals. The *Monographs* programme was subsequently expanded to include consideration of exposures to complex mixtures of chemicals (which occur, for example, in some occupations and as a result of human lifestyle factors) and exposures to biological and physical agents, such as viruses, bacteria, animal parasites, and radiation. The evaluation criteria established in 1971 have been updated at intervals by ad-hoc working groups. In 1987, these were codified to standardize the assessment of the strength of evidence for carcinogenicity to humans, and evaluation categories that would reflect these assessments were established. These criteria are to be found in the Preamble to each published volume of the *Monographs*.

The term 'carcinogenic hazard' (or, as it is called in the *Monographs* Programme, 'carcinogenic risk') is taken to mean the probability that exposure to an agent will lead to cancer in humans. Different agents may act at different stages in the carcinogenic process and several different modes of action may be involved. The aim of the *Monographs* has been, from their inception, to make qualitative evaluations of the evidence of carcinogenicity at any stage in the carcinogenic process, independently of the underlying mechanisms. Information on modes of action may, however, be used in making the overall evaluation of carcinogenic hazard to humans. As understanding of modes by which carcinogens act has advanced, mechanistic data have come to play an increasingly frequent and important role in these evaluations.

Experimental carcinogenicity data generally come from studies on rodents, usually rats and mice, and, historically, neoplasms — especially malignant neoplasms — have been the final end-point. The numbers of adequately designed, executed and described rodent carcinogenicity tests of this kind that are published have been falling in recent years, and experiments performed and published by academic investigators are now unlikely to be so-called standard two-year bioassays. Thus, the traditional source of experimental evidence for carcinogenicity on which the *Monographs* programme has historically relied is beginning to disappear, while advances in understanding chemical carcinogenesis have led to the use of short- and medium-term assays with end-points of neoplasia or lesions that are precursors to neoplasia. Evaluation of the predictive value of such studies as surrogates for lifetime studies, in which neoplasms are the end-points, is increasingly needed in the context of carcinogen evaluation and is addressed in this publication.

Another objective of this report is to attempt to define the role of data from genetic toxicology in the prediction of carcinogenic hazard. It is strongly suspected that the data obtained from the many available tests do not have equal significance in this regard. It is necessary, therefore, to attempt to distinguish the more useful tests and end-points from those that are less useful. The significance of changes in mutation frequency or specificity in cancer-related genes in tumours that arise in people and in experimental animals is also addressed. In addition to the genetic toxicological data, such information may support a particular mechanism of action.

Examples of nongenotoxic mechanisms of action that have been considered in the evaluation of the carcinogenic risk to humans of certain chemicals include receptor-mediated effects, inflammation and inhibition of gap-junctional intercellular communication, as well as certain mechanisms that are specific to single tissues or organs. These have not been considered in this document.

Section I. Assays with neoplasia or preneoplasia as the end-point and assays for cell proliferation and cell death

I.1 *Assays with neoplasia as the end-point*

There are several test models which are used in addition to the standard long-term bioassays which have neoplasia as their end-points. Some of these models have been in use for many years, such as the induction of lung adenomas in strain A mice, the induction of skin papillomas in Sencar mice, the induction of mammary tumours in rats and the induction of tumours in the newborn mouse. The potential role of more recent models is discussed here.

I.1.1 *Multi-organ models*
I.1.1.1 *Mammalian systems*
I.1.1.1.1 *Initiation–promotion*

Bioassays in which known carcinogens are used as initiators for various organs of rodents to enhance their response to subsequently administered agents are described by Shirai *et al*. (this volume). These systems yield both neoplasms and preneoplastic lesions.

Bioassays based on the concept of initiation–promotion have been developed to detect the induction of organ-specific tumours in 20–30-week protocols. In one approach, animals are given various DNA-reactive chemicals (initiators) that are known to induce cancer in different organs. A test compound is then given after the initiation procedure. In other protocols, test agents are given as either 'initiators' or 'promoters'. In two systems with different combinations of initiators studied in one laboratory, a high proportion of positive results and few false-negative responses were found for 53 compounds, irrespective of their mutagenicity (see Shirai *et al*., this volume). These systems could therefore be considered appropriate for identifying carcinogens in rodents; however, the possibility of false-positive results has not been rigorously tested.

I.1.1.1.2 *Transgenic and knock-out mice*

Transgenic animals, which have specific genes that are known to be critical to many neoplastic pathways, can be used to detect carcinogens (see Tennant *et al*., this volume). A number of transgenic and knock-out models are available. The results obtained with three — *p53*-deficient, Tg.AC (zeta globin-promoted v-Ha-*ras*) and CBF $_1$-Tg-H*ras*-2 (originally *ras*H2) — mice indicate that the models are useful. These models are appropriate for use in identifying chemicals that can induce tumours; chemicals that are not carcinogenic do not induce a response.

Heterozygous *p53*-deficient mice (with one wild-type and one null allele) on a C57BL/6 genetic background have a low spontaneous tumour incidence up to 12 months of age. In the tests conducted to date, on 19 chemicals, positive responses were obtained with all six known mutagenic carcinogens. Two noncarcinogens and 11 other chemicals, including nonmutagens, gave negative results. In general, the responses occurred in the same target tissues in a high proportion of animals, within the same dose ranges and by the same routes of administration as those used in conventional long-term bioassays. In some cases, it has been possible to identify loss of heterozygosity in the *p53* gene.

Tg.AC mice on an FVB/N genetic background carry multiple copies of a v-Ha-*ras* oncogene fused to the promoter of the mouse zeta globin gene. An important characteristic of the model is that the transgene is not constitutively expressed in the skin, and untreated skin appears normal in comparison with the skin of the wild-type FVB/N parent strain. The incidence of spontaneous skin papillomas in untreated mice is very low to zero. Thus far, 22 agents for which the results of conventional bioassays are available have been tested. Twelve of 14 carcinogens gave positive results and six of eight noncarcinogens gave negative results in a standardized protocol of dermal exposure for 20–26 weeks. Skin papilloma is the most commonly reported response, but other target sites are affected by both mutagenic and non-mutagenic carcinogens. Specific activation of the expression of the transgene is the critical event underlying responses in the model. Since induction of transgene expression can be achieved either by treatment with known carcinogens or tumour promoters or by full-thickness wounding of the skin, 'false-positive' responses might be induced by a chemical that induces some epidermal toxicity; such responses have not been observed, but the possibility should be recognized.

CBF1-Tg H*ras*-2 transgenic mice carry five or six copies of a human c-Ha-*ras* proto-oncogene which is expressed in both tumours and normal tissues. The Tg H*ras*-2 model has thus far been evaluated with at least 36 chemicals (27 carcinogens and nine noncarcinogens).

These three transgenic models have been evaluated in several laboratories, and each model has been used in at least two laboratories to test chemicals for carcinogenicity. The available data from 26-week tests indicate that these transgenic models with neoplastic end-points could be considered appropriate for identifying carcinogens and tumour promoters. Nevertheless, the limited range of chemicals evaluated to date leaves the uncertainty that agents that act through pathways other than those altered in the transgenes may not be detected, and the possibility that noncarcinogens may be identified as carcinogens.

I.1.1.2 *Nonmammalian systems*

A wide range of invertebrate and lower vertebrate species have been used as alternative models in cancer research for many years (see Bunton, this volume). Invertebrate species used in carcinogenesis bioassays include planaria and molluscs; the lower vertebrate species include amphibians and bony fish. Epizootics of neoplasia have been identified in fish and bivalves after environmental chemical contamination, and these species were subsequently used in experimental cancer bioassays. The process of cancer development in nonmammalian species and mammals is similar. Bioassays in fish species are the best developed of the nonmammalian systems, particularly with regard to hepatocarcinogenicity. Their use has shown that neoplastic development is predictable and reproducible and that the results are affected by species, age, compound and dose.

A major advantage of these models is the similarity between fish and mammals in classic cellular responses to carcinogens, such as the development of surface neoplasms after exposure to directly acting compounds and organ-specific neoplastic development after exposure to compounds that require metabolism. Other advantages are that tumours are induced in less than four to six months, preneoplastic lesions develop and the background rates of tumours are low.

The disadvantages of these models include the absence of organs that are frequent target tissues in rodents (e.g. lung and mammary gland) and difficulties in interpreting the significance of neoplasia in organs found in lower species which do not occur in mammals. Therefore, although many chemicals have been tested, particularly in fish species, the results of these assays should perhaps be combined with those of other assays.

I.1.2 *Single-organ models in mammals*

Models designed to detect neoplastic end-points in single organs have been in use for many years. These include the induction of lung adenomas in strain A mice, the induction of skin papillomas in mice, the induction of mammary tumours in female rats and initiation–promotion models in several organs in mice and rats.

In a variety of single-organ systems in rodents, agents have been tested either as 'initiators', with an organ-specific promoting agent, or as 'promoters', with an organ-specific initiating agent (see Tsuda *et al*., this volume). The most extensively used model of this type is the rat liver in which, in addition to neoplasms, preneoplastic lesions can be quantified, as discussed in section I.2.2. When data are available only on promoting activity, positive results strongly suggest the hepatocarcinogenicity of the test compound in rodents.

Similar but less extensive data are available for other systems with neoplasia as the end-point, e.g. mouse skin, mouse lung and rat stomach, kidney, urinary bladder and colon. Such studies can provide results within one year.

I.2 *Assays with preneoplasia as the end-point*

The induction of cancer by chemicals in experimental animals is well documented to be a multistep process in many tissues (see Williams, this volume). Focal preneoplastic lesions appear prior to the occurrence of neoplasms. Generally, there are more of these lesions than the subsequently developing neoplasms. While preneoplastic lesions can often be identified in conventionally stained sections, lesions that are otherwise difficult to detect can be readily identified in several tissues, such as liver, stomach and colon, by special staining techniques such as for placental-type glutathione *S*-transferase (GST-P) activity in liver foci. Such lesions have been extensively used as surrogates for neoplasms to assess the carcinogenic activity of chemicals. In other tissues, such as mouse skin, mouse lung and rat mammary gland, the progression of preneoplastic lesions to tumours is rapid and early-appearing tumours are used as the experimental end-point. The use of histologically defined preneoplastic lesions for this purpose has the advantage that the end-point occurs before the development of age-related pathological changes, including spontaneous preneoplastic lesions.

I.2.1 *Multi-organ models*

In multi-organ models in young (six-week-old) animals that have been exposed to an initiator (as described in section I.1.2), the end-point in some tissues is preneoplastic lesions which are infrequent in these animals, while neoplasia may be produced in other organs that respond to the various carcinogens used. The occurrence of preneoplasia, even in the absence of neoplasia, within a period of 20–40 weeks provides evidence of potential carcinogenic activity in rodents.

I.2.2 *Single-organ models*

A number of single-organ test systems have preneoplastic lesions as the end-point. Three widely studied models are aberrant crypt foci in the colon, pepsinogen-altered pyloric glands in the rat glandular stomach and foci of hepatocellular alteration in the liver (see Tsuda *et al*., this volume).

Foci of hepatocellular alteration can be detected in various ways, including haematoxylin-and-eosin staining and GST-P activity (see Williams, this volume). Several variations of these systems are widely used which provide reliable information within less than 30 weeks, especially when a proliferative stimulus is used. In general, few false-negative results are obtained when extended periods of administration are used. Some of the systems are designed to discriminate between initiating and promoting effects, by the use of protocols that involve a specific sequence of administration of the test agent.

In an eight-week model in Fischer 344 rats, known as the 'Ito model' (see Shirai *et al*., this volume), *N*-nitrosodiethylamine is given as an 'initiator' and, during a subsequent post-initiation stage, the rats undergo partial hepatectomy to enhance the carcinogenic process. The test compound is given during the six-week post-initiation phase. GST-P-positive liver foci are then measured and counted. Almost 300 agents have been tested in this model, and more than 90% of the nongenotoxic and genotoxic hepatocarcinogens gave positive results. A few false-negative results have been obtained, notably with peroxisome proliferators which suppress GST-P expression. Of 45 noncarcinogens, only two gave positive results. Positive results in this model are highly predictive of hepatocarcinogenic activity in rats and mice; negative results require further study.

I.3 *Assays for cell proliferation and cell death*

Disturbance of the balance between replication and death of cells is a crucial mechanism in carcinogenesis (see Schulte-Hermann *et al*., this volume). The occurrence of active or programmed cell death (often referred to as 'apoptosis') is controlled by growth regulating networks in the organism. Chemical agents may either increase replication or inhibit the death of cells or both. Enhanced replication can predispose cells to genetic changes and lead to formation of initiated cells and/or to their clonal expansion (tumour promotion). Conversely, cell death may result in elimination of initiated cells and may reverse clonal growth. Thus, although increased cell proliferation or cell survival induced by chemicals does not necessarily lead to cancer, it may indicate carcinogenic potential, whereas reversion of focal expansion after withdrawal of chemicals may indicate reversibility of mitogenic action.

Toxic doses of either genotoxic or nongenotoxic agents affect the growth of normal cells in target tissues by inducing acute or chronic injury, leading to cell death and subsequent regenerative proliferation. Primary mitogens and trophic factors stimulate cell division and/or inhibit cell death and thereby produce sustained hyperplasia in target organs. Increased proliferation is usually restricted to the early part of a prolonged treatment period. The cells in preneoplastic or neoplastic lesions may be over-responsive to mitogenic or survival signals, resulting in clonal expansion of preneoplastic lesions and their possible progression to neoplasia.

A variety of methods are available to identify proliferating cells, but there is still a paucity of specific, efficient methods to detect and quantify programmed cell death. These methods can be used as adjuncts to carcinogenicity assays, and the results can be used to facilitate the interpretation of effects and provide information about the mechanisms of the carcinogenic process. In conjunction with other biological data, this information may provide an indication of potential carcinogenicity.

I.4 *Conclusions*

- Many of the assays described above contribute to the assessment of carcinogenicity in experimental animals. In the absence of data from conventional long-term bioassays of carcinogenesis or from assays with neoplasia as the end-point, consistently positive results in several models addressing several stages in the multistage process of carcinogenesis should be considered in evaluating the degree of evidence of carcinogenicity in experimental animals.
- In well-studied models of initiation–promotion, the appearance of tumours after administration of a test chemical as an 'initiator' provides evidence of carcinogenic activity in rodents. Additional evidence of promoting activity makes the evidence compelling. When data are available only on promoting activity, the evidence is suggestive of carcinogenicity in rodents, but the information should be evaluated in conjunction with other data on the biological effects of the agent, such as genotoxicity, initiating activity and cell proliferation. When an agent has been tested for both promoting and initiating activity but has been found not to be an initiator, the evidence is less suggestive.
- In assessing the results of other well-studied models in which preneoplastic lesions are produced, positive results seen after administration of the test chemical as an 'initiator' are highly predictive of carcinogenic activity in rodents. Additional observation of promoting activity makes the evidence compelling. When data are available only on promoting activity, the evidence is suggestive, but the information should be evaluated in conjunction with other data on the biological effects of the compounds. When an agent has been tested for both promoting and initiating activity but has been found not to be an initiator, the evidence is less suggestive.
- The results of many of the studies described in this section can provide information on the mechanisms of carcinogenic activity of a chemical and its relevance to cancer in humans.

Section II. Patterns of mutations in specific genes in tumours

Most tumours evolve by a succession of genetic and epigenetic events, which include clonal selection, the acquisition of genomic instability and cell turnover. Many of these events can continue after exposure to a carcinogen has ceased. Moreover, the genome of the person or animal in which a tumour arises may be heterozygous for many genes which are polymorphic in the population, including those that affect carcinogen metabolism, and this could change the pattern of mutations that ultimately occurs in tumour-related and other genes.

Somatic mutations that are detected in cancer-related genes in tumours result from a cascade of events. Only the first of these is likely to be due to the action of a carcinogenic agent. Changes in DNA reflect the intracellular availability and metabolism of the potential damaging agent. Although changes in DNA structure resulting from such interaction may persist through subsequent cell divisions, these changes will inevitably be subject to the modifying influence of biological filters, including DNA repair mechanisms and differential selection of some structural changes in preference to others. These factors may distort the pattern of changes that initially result from DNA–carcinogen interaction, so that what is finally detected in tumour cells is only partly consistent with the chemistry of the initial events. Caution must therefore always be exercised in attempting to infer the identity of a carcinogen from its 'footprints' in tumour cell DNA.

The more genetically diverse the population in which tumours arise, the more difficult it is to discern a specific pattern of gene mutation ('signal') that is due to a putative carcinogenic agent within the 'noise' that includes all mutations that arise from other causes. In principle, therefore, it should be more straightforward to minimize the background and to detect consistent exposure-related mutation patterns in a given gene in inbred, genetically homogeneous populations. Such populations exist among laboratory mice and rats, but not in human societies. Therefore, conclusions about causal relationships between exposure to carcinogens and mutation patterns can generally be drawn more easily from the results of experiments in animals.

II.1 ras *Mutations in experimental tumours*

During the past decade, *ras* mutation frequency and spectra have been catalogued in three members of the *ras* gene family (H-, K- and N-*ras*) in certain naturally occurring tumours in in-bred rats and mice and in their F_1 hybrids. These tumours include many kinds that are also commonly seen in animals that have been exposed to carcinogens. The patterns and frequencies of *ras* mutations in these naturally occurring tumours have been determined with a high degree of precision and provide a basis for comparison with carcinogen-induced tumours of the same kinds. Exposure-related changes in *ras* mutations may be qualitative, presenting a different mutation spectrum from that of naturally occurring tumours of the same site and type, or quantitative, presenting an increase or decrease in the fraction of tumours containing *ras* mutations.

The existence of mutations in a *ras* gene in tumour cells implies the participation of that gene in the pathogenesis of that tumour. A change in the fraction of tumours that contain *ras* mutations, in comparison with the fraction seen in comparable tumours in untreated animals, suggests an exposure-related effect (see Sills *et al*., this volume). Genotoxic carcinogens often increase the frequency of *ras* mutations, generally in a specific member of the *ras* gene family. In contrast, in mouse liver, there is evidence that nongenotoxic carcinogens, including phenobarbital, oxazepam and several chlorinated hydrocarbons, give rise to tumours in which the *ras* mutation frequency is the same as or lower than that of naturally occurring tumours.

The changes in the pattern of *ras* mutations in response to a given agent also help to confirm that the agent caused the tumour. Furthermore, the *ras* mutation frequency and spectra can differ at low and high doses of a carcinogen: e.g. liver tumours induced in mice by *N*-nitrosodiethylamine, Harderian gland neoplasms induced in mice by isoprene and mammary gland tumours induced in rats by *N*-nitroso-*N*-methylurea.

The Working Group noted the following limitations in the use of *ras* gene mutations for identifying carcinogens in experimental animals:

- The database for *ras* mutations in most experimental tumours remains small. The largest amount of data is available for liver, lung and skin neoplasms in mice, but the database is much smaller for tumours at other sites in mice, such as the Harderian gland, forestomach and kidney and for all sites in rats.
- The *ras* genes do not appear to be mutated in some types of tumours, such as those of endocrine organs, that occur frequently in bioassays.
- In many reported studies, information on naturally occurring tumours is not available.
- It may be useful to continue to expand the database on *ras* mutations, examine dose–response relationships for *ras* mutations and confirm that nongenotoxic carcinogens induce tumours that do not contain mutations in *ras* genes at other sites in rats and mice.

II.2 *Genes other than* ras *in experimental tumours*

Although data exist on members of the *ras* gene family in experimental tumours, information on mutations in other oncogenes or in tumour suppressor genes from experimental tumours is scarce. Moreover, few comparative studies have been conducted on the sites and nature of mutations in tumours induced by different agents, and in naturally occurring tumours. Those analyses that have been made have been limited in most cases to only a portion of the coding exons. Virtually no reports on structural changes at other sites, such as promoter regions, that might indirectly affect gene expression and gene product function, have been published.

The available data demonstrate that genes involved in the pathogenesis of a given neoplasm in humans are not necessarily involved in comparable neoplasms in experimental animals. Moreover, the exon/intron organization of specific genes may vary between species, and processed pseudogenes containing highly but imperfectly homologous and nonfunctional DNA sequence may exist in some species, but not others, and may simulate the existence of point mutations. Analysis of *p53* in mice and rats is complicated by both these considerations.

Evidence for a role in the pathogenesis of at least some rodent tumours has been summarized for the transforming genes *neu*, *raf*, *ret* and TGF-*a*, the tumour suppressor genes *p53*, *apc*, *p15/p16*, *Rb*, *vhl* and *wt1* and the *DCC* locus (see Perantoni & Rice, this volume). Much of this evidence is derived from genetically engineered constructs that differ significantly from the normal organization of the genes. Somatic cell mutations in *neu*, *p53* and *apc* in certain carcinogen-induced tumours in rats and mice are consistent with a direct effect of the carcinogens involved, but the data are in general too limited to suggest patterns of mutation that would be usefully indicative of the action of a class of carcinogenic agents, much less of specific chemicals within a class.

Despite these limitations, the demonstration that mutations in any of these genes are present in tumour cells is useful information that should be noted in evaluating possible carcinogenicity; however, the paucity of the literature on mutation patterns in these genes in naturally occurring tumours severely limits the use of such data to distinguish naturally occurring from chemically induced tumours.

II.3 *Mutations in cancer-related genes in humans*

Unlike inbred experimental animals, humans are genetically heterogeneous. Furthermore, they are usually exposed to complex mixtures of chemicals, and only limited qualitative and quantitative information is available on specific agents. Of the many genes involved in human cancer, only a small number show evidence of mutations associated with environmental or chemical exposure. These genes include *ras*, *p53*, *p16*, *PTCH* and *VHL*. A large database is accumulating, however, only for *ras* and *p53* and in only a limited number of cancer types. Three criteria should be applied in determining whether a particular mutation profile can be considered to be a footprint of exposure:

- The suspected exposure factor should generate a characteristic DNA signature in experimental assays of genotoxicity.
- The same DNA signature should be detectable in a significant proportion of tumours from putatively exposed individuals, as compared with tumours of the same kind in unexposed people.
- The same DNA signature should already be detectable at low levels either in normal tissues at the site of the tumour or in preneoplastic or early lesions in exposed individuals.

At present, these criteria have been met for *p53* mutations in liver cancers associated with exposure to aflatoxin B_1, *p53* in squamous-cell skin tumours associated with exposure to ultraviolet radiation and K-*ras* and *p53* in lung tumours associated with tobacco smoking (see Devereux *et al*. and Hernandez-Boussard *et al*., this volume).

A major challenge is to collate information reported in the literature on cancer gene mutations, on genetic susceptibility and on individual exposures. Indeed, most of the information published on mutations in cancer-associated genes relates to cases of disease selected on the basis of clinical or pathological relevance rather than on reliable, molecular epidemiological criteria. Moreover, the inherent biological heterogeneity of the material analysed is often compounded by great variability in the way personal characteristics, exposures and diseases are described for individual cases. In the future, it will be necessary to promote two parallel approaches to reduce this heterogeneity. One is to develop detailed, flexible databases to link descriptions of mutation with individual histories, including detailed, specific information on exposure. The other is to perform large-scale molecular epidemiological studies on cancer-related genes and susceptibility genes in well-defined populations. Such studies have been hindered so far by technical considerations, but the development of rapid, cheap methods for analysing mutation is opening new possibilities. The development of databases and the conduct of molecular epidemiological studies require implementation of appropriate guidelines for ethical conduct (see Schulte *et al*., 1997).

Section III. Use of genetic and related effects in experimental systems and human exposures in evaluating carcinogenicity

Introduction

Genetic changes resulting from gene mutations, alterations in chromosomal structure and number and gene amplification are associated with numerous types of tumours. Most of the chemicals classified as carcinogenic or probably carcinogenic to humans by working groups convened by IARC have been shown to be mutagenic in mammalian cells *in vivo* and/or *in vitro* (Waters *et al*., this volume), providing strong evidence that mutagenicity plays an important role in the activity of most carcinogens.

The mutagenic events involved in carcinogenesis are produced by one of two broad modes of action. The first may involve modification of DNA or chromatin by an agent or its metabolites, causing gene or chromosomal mutations, or by interference with other processes, such as gene transcription or spindle function. The second process involves perturbation of homeostasis, which may be mediated by tissue necrosis, apoptosis, or cellular turnover, leading indirectly to the expression of mutations in DNA. Consideration of information on genetic and related effects in the context of the *IARC Monographs* programme has been used to evaluate a number of chemicals (see Table III.1).

The characteristics of the results of short-term tests that allow assignment of relative strength for the purposes of predicting carcinogenicity include: the phylogenetic proximity of the test organism to humans, the degree of involvement of the end-point in the process of carcinogenesis and how predictive the results of a test are for the carcinogenicity of the particular class of chemicals under consideration. The terms used to describe the performance of screening tests and screening programmes are illustrated in Table III.2. Reproducibility and statistical power are particularly important attributes when the level of mutant induction is low. While sensitivity and specificity are useful for assessing the predictive value of a given test for defining carcinogenic potential, these measures are inappropriate for comparing the performance of assays unless exactly the same chemicals have been evaluated.

The following sections identify the various types of short-term tests used for detecting genotoxicity and cell transformation, with discussion of their potential contribution to assessing carcinogenic hazards for humans.

III.1 *DNA damage and repair*

A number of tests are available for measuring DNA damage and repair (Table III.3).

III.1.1 *DNA adducts*

DNA adducts are known to be a cause of mutations and are associated with mutations in oncogenes. Accordingly, they are considered to be involved in the carcinogenicity of some agents.

Two techniques are currently used for the ana-lysis of DNA damage. Chemical-specific techniques rely on knowledge of the structure and properties of the DNA adducts to be detected and are often orientated towards the measurement of DNA damage in situations in which the exposure is well characterized. Non-specific techniques have minimal requirements such as a covalent modification of DNA to perturb the function of an enzyme (as in the case of the nuclease P1 version of ^{32}P-postlabelling) or the introduction of a detectable property such as radioactivity (in minute quantities) into DNA, as performed for investigations by accelerator mass spectrometry. Current analytical methods allow the detection of adducts in small quantities of DNA (less than 1 μ g) extracted from human and animal tissues (see Shuker, this volume).

Although relatively few studies have been carried out in which experimental animals were exposed to low doses of carcinogens and both the levels of relevant DNA adducts in target organs and tumour outcome have been measured, a clear relationship is seen between these two parameters. This relationship (linear in the available data) holds only at very low doses, approaching those to which humans are exposed; over the whole dose range, the shape of the dose–response curve is usually far from linear. On the basis of these experimental results, increased levels of specific DNA damage may be predictive of increased incidences of tumours in groups of people.

Certain DNA modifications, such as those caused by reactive oxygen species, may originate from both exogenous and endogenous sources, and the levels detected reflect the net effect of all routes of exposure and repair. In general, DNA adducts represent recent exposure, as distinct from a cumulative measure, which may be derived from the quantification of mutations.

III.1.2 *Assays for DNA damage and repair*

Assays for DNA damage and repair have been developed either for determining general effects on DNA or for assessing effects in target tissues. The bacterial chromotest assay, for example, assesses the ability of a chemical to induce DNA damage (see Quillardet & McGregor, this volume). Alkaline elution and unscheduled DNA synthesis assays have been used extensively to determine repair of induced DNA damage *in vivo* and *in vitro*. It should be noted that a positive response in some of these assays is highly predictive of carcinogenicity in rodents, whereas a negative response is of little significance.

The single-cell gel electrophoresis, or Comet, assay can detect double-strand breaks at neutral pH and single-strand breaks at high pH, alkali-labile damage and incomplete excision repair. Its advantage is that it can be used to determine DNA damage in individual target cells in any organism. While the current database for assessing the predictivity of the Comet assay for carcinogenic potential is relatively small, particularly for noncarcinogens, it is growing rapidly. The assay is highly sensitive for carcinogens, but there is currently little information from which to assess its specificity (see McGregor & Anderson, this volume).

III.2 *Gene mutations*

III.2.1 *Bacteria*

A number of tests for mutagenicity involve the use of bacteria. The *Salmonella*/microsome reverse mutation assay (Ames test) is the most extensively used and best validated of these tests. The success of this test has been due to the construction of bacterial strains that are very sensitive to mutagenic chemicals and to the addition of an exogenous metabolic activation system from rat liver, to mimic the metabolism of the chemicals in mammals. Considerable reliance can be placed on the *Salmonella*/microsome test as a predictor of carcinogenicity if the result is positive, but not if it is negative; however, the performance of the test for the particular class of chemicals must be considered (Quillardet & McGregor, this volume).

III.2.2 *Fungi*

Yeasts and other fungi provide test systems of potential value in both the detection and study of the mechanisms of action of chemical carcinogens (see Parry, this volume). Of these, only systems involving the yeast *Saccharomyces cerevisiae* have been adequately evaluated for their ability to detect chemical carcinogens in comparative studies with coded chemicals of defined activity in cancer assays in rodents. A positive result with a potential carcinogen in a yeast nuclear mutation assay should be considered indicative of a mutagenic mode of action; however, the mitochondrial mutation end-point has not been validated and its significance in carcinogen prediction is limited.

The use of fungal assays can contribute to an understanding of the mechanism of action of a genotoxic carcinogen because of the broad range of genetic end-points that are available for study.

Table III.4 includes an overview of the current status and suitability of fungal end-points for the study of chemical carcinogens.

III.2.3 Drosophila melanogaster

The largest database from genotoxicity studies in *Drosophila* is available on sex-linked recessive lethal forward mutations and includes data on 700–750 chemicals. The database for the consequences of chromosomal breakage is much smaller, about 100 chemicals having been tested for reciprocal translocations and/or chromosome loss. Comparative studies showed that only 41% of 66 chemicals that induced sex-linked recessive lethal mutations also induced reciprocal translocations. Studies of differential sensitivity to forward mutation and to reciprocal translocation and chromosomal loss showed that the dose of chemical mutagens and the site of alkylation affect the type and frequency of genetic damage induced in *Drosophila* (see Vogel *et al*., this volume).

One strength of the test for sex-linked recessive lethal mutation is its high specificity, which is close to 1. Thus, whereas a negative response in *Drosophila* has little meaning with respect to the potential genotoxicity of a chemical, a positive response (mutation frequency equal or greater than 5 times the control level) provides good evidence that a chemical is a mutagen and a carcinogen in other species.

Loss of heterozygosity of the reporter genes is the basic principle of assays for recombination in *Drosophila*. The better performance of these tests than of germ-line assays appears to be the result primarily of three factors: the application of multiple dose protocols, the use of distinct tester strains with heterogeneity for activation of procarcinogens and the fact that they detect a broader range of genetic alterations. A full evaluation of the entire database for the somatic mutation assays has not yet been conducted and is therefore recommended. The crux of this evaluation will be to see whether their sensitivity is greater than that of the assay for sex-linked recessive lethal mutations while, at the same time, the high specificity of the latter *Drosophila* assays is retained.

III.2.4 *Mammalian cells*

Because mammalian somatic cells are diploid, most genetic changes caused by exposure to genotoxic agents do not result immediately in a phenotypic change, unless they involve dominant or X-chromosomal mutations. Thus, a limited number of target genes is available. Selection procedures have been developed for a limited number of target genes which allows detection of the frequency of mutations in mammalian cell lines, skin fibroblasts from rats, T lymphocytes from rodent spleen and T lymphocytes from human blood. For several of these marker genes, mutational assay systems have been set up to screen potential genotoxic chemicals (see Table III.5). The majority of the data are derived from studies in cell lines (see van Zeeland & Vrieling, this volume).

The DNA sequence of some target genes is known and, therefore, the spectrum of mutations caused by exposure to a genotoxic agent can be identified by DNA sequencing. Some of these genes, such as *hprt* and *aprt*, are sensitive to many different types of base-pair change; thus the mutational spectrum in these genes reflects the spectrum of unrepaired mutagenic DNA damage caused by the exposure. Indeed, the mutational spectra induced by ultraviolet light, alkylating agents or active metabolites of benzo[*a*]pyrene show clear characteristics of the nature of the DNA damage induced. Furthermore, by comparing spectra obtained in normal cells with those observed in cells deficient in a specific DNA repair pathway, the contribution of specific types of DNA damage to mutation induction has been identified. Therefore, mutational spectra in a number of marker genes reflect the characteristics, or show a fingerprint, of the genotoxic agent used to induce the mutations. Distinct spectra of mutations in the *p53* gene have been found in specific human tumours and in tumours of rodents treated with defined carcinogens, namely ultraviolet radiation, benzo[*a*]pyrene and aflatoxin B_1. In both situations, these mutations are consistent with the chemistry of the induced DNA damage and with the mutations observed in cultured cells exposed to these agents. Similar data are not currently available for other agents.

The action of DNA repair processes clearly affects mutational spectra. Animal models with altered DNA repair genes are therefore suitable for comparing the mutational spectra in target genes with those in genes mutated in tumours and for analysing the relative contributions of the different types of DNA damage responsible for the mutagenic response.

Some defective DNA repair systems are known to be responsible for a mutator phenotype, in which a number of mutations are generated in a single cell. Since mutations induced by such genomic instability are not necessarily a direct result of exposure to mutagens, and since mutagens can induce more mutations in cells with a mutator phenotype, genomic instability may confound interpretation of the mutagenicity of agents.

III.2.5 *Mutation in transgenic animals*

Several transgenic models of animals carrying various specific reporter genes have been developed and are being used to investigate in-vivo mutagenesis. While other systems are still in various stages of development and, therefore, only limited data are currently available, the two rodent gene mutation assays based on λ shuttle vector systems have been explored by a number of investigators (see Schmezer & Eckert, this volume). These systems can detect base substitutions, frameshift mutations and insertions, but only small deletions of up to approximately 7.5 kilobases.

More than 50 chemicals and various types of radiation have been analysed in the *lacI* (BigBlue™) and/or the *lacZ* (Muta™Mouse) system. Most of the studies have been performed with three mouse strains (*lacI*: C57BL/6 and B6C3F$_1$; *lacZ*: BALB/c x DBA/2), while few studies have been published of transgenic *lacI* Fischer 344 rats, which are a recent development. The wide spectrum of organs and tissues in which mutations can be analysed is a major advantage of these transgenic rodent assays. They can also provide a useful tool for investigating mutation induction mediated through in-vivo processes such as inflammation.

A further advantage of the *lacI*/*lacZ* transgenic rodent mutation assays is that DNA sequencing can be used to analyse mutational spectra. The mutational spectra contribute to the study of mutagenesis in two respects: they can provide additional proof for specific mutagenic changes when only a weak overall response is obtained and the mechanisms of induced mutations may be elucidated by identifying the specific DNA sequence alterations.

Carcinogens have been shown to have mutagenic activity at the doses used in chronic long-term bioassays in rodents. It has been suggested that assays in transgenic rodents given the maximum tolerated dose used in cancer bioassays would provide data on mutations of immediate relevance to cancer. Test compounds can show mitogenic effects at certain doses. On the basis of the assumption that the mitogenic activity of a tissue is correlated with its mutation fixation time, possible mitogenic properties of the test compounds should be taken into account.

No noncarcinogenic chemicals have so far been reported to give positive results in the assay, but few have been studied and the protocols for the assays in transgenic rodents are still undergoing development.

III.3 *Chromosomal changes in mammalian systems*

All tumours contain chromosomal alterations. Specific small deletions, translocations or aneuploidy are involved in specific steps in tumour development. Nonspecific alterations are a characteristic of the genomic instability that is a feature of tumours.

The chromosomal changes that are measured in short-term assays are structural, numerical alterations and/or sister chromatid exchanges (Table III.6). Structural changes are produced by errors of DNA repair or errors in DNA replication. The former is the primary mode of action of ionizing radiation, radiomimetic chemicals and agents that can interact with DNA–enzyme complexes, such as topoisomerases. Errors in DNA replication are the mode of action of most chemicals that can form adducts with DNA.

Chromosomal numerical changes are produced by errors in chromosome segregation caused by either failure of sister chromatids to segregate correctly or failure of a chromosome to leave the metaphase plate. Lagging chromosomes and acentric chromosome fragments that are excluded from daughter nuclei lead to the formation of micronuclei. Chemicals that affect the mitotic spindle are the class currently also known to induce aneuploidy. Sister chromatid exchange is produced by errors in DNA replication on a damaged DNA template, perhaps at the site of a stalled replication fork. The various modes of action must be considered in the interpretation of assays to detect various cytogenetic end-points.

Until recently, most short-term assays have measured the frequencies of nontransmissible (cell lethal) aberrations, such as large deletions and dicentrics. Recently developed fluorescent in-situ hybridization methods allow assessment of transmissible chromosomal aberrations, such as reciprocal translocations and inversions. Transmissible aberrations are of much greater relevance for hazard identification. Unstable aberrations are perhaps best considered as indicators that stable ones have also been induced, in the absence of their direct observation.

As a general rule, the most informative in-vitro assays are those involving either primary cells that maintain some metabolic competence and a stable karyotype or cultured human cells that contain transfected human metabolic enzymes and have a stable karyotype.

In-vivo assays are more appropriate for linking clastogenicity and cancer, especially after medium-term exposure, when transmissible aberrations are assessed. The induction of chromosomal aberrations in bone marrow is considered highly predictive of carcinogenicity. The presence of sister chromatid exchange should be viewed as an indicator of exposure and not a mutagenic response.

Monitoring of cytogenetic alterations in peripheral lymphocytes from human populations is a special type of in-vivo assay (Preston, this volume). Generally, the chromatid-type aberrations or sister chromatid exchanges are produced during the S phase in culture, and their frequency reflects only that amount of DNA damage that remains at the time of the in-vitro replication. Thus, with a few exceptions, only recent exposure is assessed. Further, exposure– (or dose–) response assessments should be part of any monitoring study, and careful attention should be paid to epidemiological aspects, with particular attention to confounders of response. With current technical advances, transmissible chromosomal aberrations can be measured that indicate a cumulated response due to long-term exposure. The available data from exercises in human cytogenetic monitoring are not simple to interpret, and their usefulness for conclusions beyond an indication of exposure must be considered with caution.

III.4 *Cell transformation*

Extensive research has shown that the cellular changes that occur during cell transformation *in vitro* closely parallel multistage chemical carcinogenesis *in vivo* (see LeBoeuf *et al*., this volume). Thus, alterations in the control of cell differentiation and replication occur in a stepwise manner, resulting in autonomous cellular growth. Several transformation systems have been used (Table III.7). The genetic changes associated with the phenotypic changes have been characterized in several instances and are consistent with oncogene activation and loss of suppressor gene function. Collectively, the existing data strongly support the biological relevance of cell transformation to in-vivo carcinogenesis and, in that respect, make it a logical approach for predicting the carcinogenic potential of chemicals and mechanisms of action.

Of the transformation assays used at present, the largest databases exist for those involving BALB/c-3T3 and Syrian hamster embryo cells. Analyses of these databases indicate that these assays are highly sensitive and specific for detecting the carcinogenic activity of chemicals. Previous analyses of the BALB/c-3T3, Syrian hamster embryo and C3H 10T1/2 assays for their ability to detect human carcinogens demonstrated a sensitivity of approximately 70–80%. Importantly, carcinogenic agents that act through a variety of mechanisms, including those considered as 'nonmutagenic', are detected with these assays.

Conclusions

Most authorities have come to the view that a minimal set of data suffices to define a chemical as a mutagen/nonmutagen. These data usually consist of gene mutations in bacteria and mammalian cells and in-vitro and in-vivo cytogenetics. Past experience has shown that data for certain types of genetic and related effects, which are commonly summarized in the Monographs, are not suitable for classifying or predicting carcinogenic hazard. Newer assays which could provide additional information include the Comet assay, mutations in transgenic animals, fluorescent in-situ hybridization and cell transformation.

References

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Table III.1. Genetic and related effects and other factors considered in the overall evaluation of 12 chemicals assessed in recent *IARC Monographs*

Chemical/ Monograph vol.	Evaluation		GRE factors	Other factors
	H	**A**		
	Group 1			
2,3,7,8-TCDD[a] Vol. 69	L	S		Multisite carcinogen in experimental animals that acts through mechanisms involving the Ah receptor, which also occurs in humans. Tissue concentrations similar in heavily exposed humans and in rats given carcinogenic doses.
Ethylene oxide Vol. 60	L	S	Powerful mutagen/clastogen at all phylogenetic levels; germ cell mutagen; persistant, dose-related increase in CA and SCE in lymphocytes and MN in bone marrow of workers; haemoglobin adducts in humans and DNA and haemoglobin adducts in rodents	Associated with malignancies of lymphatic and hematopoietic system in humans and animals
	Group 2A			
1,2,3-TCP[b] Vol. 63	ND	S	Mutagenic to bacteria and mammalian cells *in vitro*; DNA adducts *in vivo*	Multi-site, high tumour incidence in mice and rats; metabolites similar in human and rodent microsomes
Acrylamide Vol. 60	I	S	DNA adducts in rodents and haemoglobin adducts in humans and rodents; germ and somatic cell effects *in vivo*; gene mutation, CA, and transformation *in vitro*	–
Styrene-7,8-oxide Vol. 60	ND	S	DNA adducts in humans and rodents; gene mutation in bacteria and in rodent cells; CA, MN, and SCE in human cells *in vitro*; CA and SCE in mice *in vivo*	–

Table III.1 (contd)

Chemical/ Monograph vol.	Evaluation		GRE factors	Other factors
	H	**A**		
MOCA[c] Vol. 57	I	S	Genotoxic in bacteria and mammalian cells; DNA adducts (adducts in target tissues for carcinogenicity in animals same as those in urothelial cells from exposed workers)	Metabolized to N-hydroxy-MOCA in humans, rats and dogs
IQ[d] Vol. 56	I	S	Genotoxic *in vivo* and *in vitro*	Human microsome metabolites cause DNA damage in bacteria
Diethyl sulfate Vol. 54	I	S	Ethylates DNA; positive in virtually all tests examined; potent effects in mammalian somatic and germ cells *in vivo*	–
1,3-Butadiene Vol. 54	L	S	Epoxy-metabolites interact with DNA; base-substitution mutation in bacteria similar to mutation in the K-*ras* oncogene in mouse tumours	Metabolism *in vitro* is qualitatively similar in humans and animals
Captafol Vol. 53	ND	S	Active in wide range of tests including rat dominant lethal test	–

Table III.1 (contd)

Chemical/ Monograph vol.	Evaluation		GRE factors	Other factors
	H	**A**		
	Group 2B			
Vinyl acetate Vol. 63	I	L	Vinyl acetate and acetaldehyde are genotoxic in human cells *in vitro* and in animals *in vivo*	Transformed to acetaldehyde in human blood and animal tissue; acetaldehyde is carcinogenic in animals
Styrene Vol. 60	I	L	CA in human blood *in vitro*; CA, SSB, and DNA and haemoglobin adducts observed in workers exposed to styrene in the reinforced plastics industry	Metabolized to styrene-7,8-oxide which binds to DNA and is genotoxic *in vivo* and *in vitro*; styrene-7,8-oxide in blood of workers exposed to styrene.

S, sufficient; L, limited; I, inadequate; ND, no data; H, humans; A, animal; CA, chromosome aberrations; SCE, sister chromatid exchange; MN, micronuclei; SSB, single-strand breaks

[a] 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzo-*para*-dioxin

[b] 1,2,3-T, 1,2,3-trichloropropane

[c] MOCA, 4,4'-methylene bis(2-chloroaniline)

[d] IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline

Table III.2. Terms used to describe screening tests and screening programmes

Test outcome	Carcinogen		
	Yes	**No**	**Total**
Positive	a	b	a + b
Negative	c	d	c + d
Total	a + c	b + d	N = a + b + c + d

Term	Definition
Sensitivity	a/(a + c)
Specificity	d/(b + d)
Positive predictivity (predictive value)	a/(a + b)
Negative predictivity	d/(c + d)
Accuracy (concordance)	(a + d)/N

Adapted from Cooper *et al.* (1979)

Table III.3. DNA damage and repair assays	
End-point	**Potential use for detection of chemical carcinogens**
Adducts	
DNA adducts	Identification of human exposure and/or agents
Haemoglobin adducts (as a surrogate for DNA)	Internal dose measure
Non-specific assays (e.g., ^{32}P-postlabelling, accelerator mass spectrometry)	Internal dose measure
Chemical-specific assays (e.g. immunoassays)	Internal dose measure
DNA strand breakage	
Elution assay	
	False-positives obtained when cytotoxicity is not measured. High sensitivity and high specificity reported *in vitro*, small in-vivo database
Comet assay	
Double-strand breaks (neutral pH), single-strand breaks alkali labile abasic sites, incomplete excision repair, DNA–DNA cross-linking (pH > 13) *in vitro* and *in vivo*, including humans	Generally not used for predicting carcinogenicity; used for clinical monitoring and investigations of mechanisms *in vitro* and *in vivo*; high sensitivity for carcinogens, little known about its specificity
DNA repair	
Differential toxicity	
Bacillus subtilis rec A$^{+/-}$ *Escherichia coli pol* A$^{+/-}$	Generally used as a prescreen and not for hazard evaluation; qualitative assay
Inducible genes	
Escherichia coli Chromotest, *umu* test; prophage induction	Generally used as a prescreen. Positive results should be treated in the same way as positive results in the *Salmonella*/microsome test.
Unscheduled DNA synthesis (UDS)	
Labelled thymidine incorporation during non-semi-conservative DNA synthesis, usually in hepatocytes *in vitro* and *in vivo*, only rarely including humans	Positive results from the in-vitro or the in- vivo UDS assays are highly predictive for rodent carcinogenicity, but a negative result has no predictive value.

Table III.4. Overview of non-mammalian test systems for the detection of heritable genetic alterations	
Test system	**Type of genetic alteration**
Prokaryotic systems	
Salmonella typhimurium	Reverse mutation (Ames test)
Salmonella typhimurium	Forward mutation
Escherichia coli	Reverse and forward mutation
Lower eukaryotic systems	
Saccharomyces cerevisiae	Forward and reverse mutation, recombination, chromosome malsegregation
Schizosaccharomyces pombe	Forward and reverse mutation
Aspergillus nidulans	Forward and reverse mutation, chromosome malsegregation
Neurospora crassa	Forward and reverse mutation, recombination, chromosome malsegregation
Drosophila melanogaster	
Sex-linked recessive lethal mutation	Base substitutions and small aberrations
Heritable translocation	Reciprocal chromosomal aberrations
Somatic recombination and mutation	Loss of heterozygosity by recombination of various types, mutation and structural aberrations

Table III.5. Marker genes used for the detection of gene alterations in mammalian cells

Gene	Organism	Cell type	Molecular analysis possible	End-point	
tk	Mouse	Lymphoma cells ($tk^{+/-}$) in culture	+	BP changes, deletions	
tk	Hamster	CHO cells ($tk^{+/-}$) in culture	+	BP changes, deletions	LOH
hprt	Hamster	CHO or V79 cells in culture	+	BP changes, deletions	
hprt	Rat	Skin fibroblasts (granuloma pouch)	+	BP changes, deletions	
hprt	Rat	Liver epithelial	+	BP changes, deletions	
hprt	Mouse	T lymphocytes from the spleen	+	BP changes, deletions	
hprt	Humans	T lymphocytes from peripheral blood	+	BP changes, deletions	
aprt	Hamster	CHO cells ($aprt^{+/-}$) in culture	+	BP changes, deletions	LOH
aprt	Mouse	T lymphocytes from spleen of $aprt^{+/-}$ mice	+	BP changes, deletions	LOH
aprt	Humans	T lymphocytes from peripheral blood of $aprt^{+/-}$ individuals	+	BP changes, deletions	LOH
HLA	Humans	T lymphocytes from peripheral blood	+	BP changes, deletions	LOH
Na^+/K^+ ATPase	Hamster	CHO or V79 cells in culture	-	BP changes	
DLB-1	Mouse	Small intestine	-	BP changes, deletion (?)	
lacI	Mouse/rat	Multiorgans (BigBlue™)	+	BP changes, small deletions	
lacZ	Mouse	Multiorgans (Muta™Mouse)	+	BP changes, small deletions	

BP, base pair; LOH, loss of heterozygosity

Table III.6. Assays of observable chromosomal alterations	
End-point	**What is measured**
Stable chromosomal aberrations (metaphase or interphase analysis by fluorescent in-situ hybridization)	Transmissible chromosomal damage (reciprocal translocations/inversions)
Unstable chromosomal aberrations (metaphase analysis)	Non-transmissible chromosomal damage (dicentrics/large deletions)
Micronuclei	Acentric chromosomal fragments (centromere-negative) Chromosomal loss (centromere-positive)
Numerical chromosomal changes (metaphase or interphase analysis)	Non-disjunction, chromosomal loss
Sister chromatid exchange	DNA damage leading to replication errors (indicator of exposure)

Table III.7. Cell transformation assays for predicting carcinogenic potential			
Transformation assay	**Cell type used**	**End-point measured**	**Assembled data for assessing assay predictivity**
Syrian hamster embryo	Finite life span hamster embryo	Morphologically transformed colonies	++++
BALB/c-3T3	Immortalized mouse cell line	Foci	++++
C3H 10T1/2	Immortalized mouse cell line	Foci	++
RLV	Virally infected rat cell line	Foci	+
SA7	Virally infected rat cell line	Foci	+