Chapter 9: Genotoxicity and Carcinogenicity

Introduction

A chemical is considered to be mutagenic if it is capable of inducing heritable changes (mutations) in the genotype of a cell as a consequence of alterations to, or loss of, genes, chromosomes or parts of chromosomes. Genotoxicity is a broader term that refers to the ability to interact with DNA and/or the cellular apparatus that regulates the fidelity of the genome, such as the spindle apparatus and topoisomerase enzymes (1).

A chemical is regarded as carcinogenic if it induces cancer (or increases the incidence of cancer formation). The process of carcinogenesis is considered to result from the transition of normal cells into cancer cells by way of a sequence of stages associated with alterations in rates of cell growth and death, changes in cell differentiation, and the invasion of healthy tissues by cancer cells. Genotoxic carcinogens induce cancer by a direct interaction of the chemical (or its active metabolite) with DNA, whereas non-genotoxic carcinogens exert their carcinogenic effects through other mechanisms.

Current Approaches to Genotoxicity Testing

Genotoxicity testing is an important part of the hazard assessment of chemicals for regulatory purposes (2, 3). It is undertaken for two main reasons (4, 5): a) to detect chemicals that might cause genetic damage, including point mutations, in germ cells, and thus increase the burden of genetic disease in the human population; and b) to detect chemicals that might be carcinogenic (based on the assumption that mutagenesis is a key event in the process of carcinogenesis). A scheme for genotoxicity testing is presented in Figure 9.1.

The use of *in vitro* assays in regulatory genotoxicity testing

The standard regulatory approach to genotoxicity testing is to use a tier-testing scheme comprising at least two *in vitro* tests in the first level of the tier: a bacterial mutagenicity assay (OECD Test Guideline [TG] 471; usually the Ames test with *Salmonella*, but sometimes with *Escherichia coli*) and a cytogenetics assay (OECD TG 473; usually a metaphase analysis either with human lymphocytes or with rodent cell lines; 6–9). Two different endpoints are required, since chemicals can induce gene mutations and/or chromosomal damage. It is important to realise that these initial tests are used as a means of trying to establish the potential of a chemical to elicit a mutagenic effect, and that it is customary, particularly with drugs and food additives (i.e. where there is a likelihood of extensive and prolonged human exposure), to demonstrate that the mutagenic potential detected *in vitro* is realised in an intact animal. In other words, and contrary to hierarchical testing schemes for other types of toxicity, a negative result *in vitro* is usually considered sufficient to indicate lack of mutagenicity, whereas a positive result is not considered sufficient to indicate that the chemical represents a mutagenic hazard.

Some regulatory agencies encourage the use of the mouse lymphoma assay instead of a cytogenetics test, because this assay detects both point mutations and chromosomal damage. However, it is a difficult assay to perform, and it is necessary to conduct colony sizing accurately, in order to be able to assess the ability of a chemical to induce chromosomal damage.

It is also possible to use genetically engineered cell-lines, expressing one or more phase I and phase II enzymes, to detect a variety of genotoxic endpoints caused by chemicals requiring metabolism (10–13). These have the advantage over exogenous metabolising systems that metabolites are generated intracellularly, i.e. close to the target DNA.

The use of *in vivo* assays in regulatory genotoxicity testing

The usual approach in *in vivo* genotoxicity testing is to look for cytogenetic damage in the bone-marrow by using either the micronucleus assay or metaphase analysis. *In vivo* tests are used to determine whether the potential for genotoxicity detected *in vitro* is realised *in vivo* (i.e. to identify false-positives). False-positives *in vitro* can have a variety of causes, such as preferential metabolic activation and excessively high dose levels, without transport away from the target site.

Despite the problems inherent in interpreting the data produced (see below), a negative result is often taken to imply that the chemical is unlikely to be either a germ-cell mutagen or a genotoxic carcinogen, although further assessment, possibly culminating in a rodent bioassay, is sometimes required. A positive result in the bone-marrow is interpreted as being indicative of genotoxicity to the germ cells (as there are no known germ cell mutagens that are not somatic cell mutagens), and also of carcino-

Figure 9.1: A tiered testing strategy for genotoxicity

Step		Result
1. Use a validated (Q)SAR(s) for genotoxicity ↓	→	*positive or negative result*[a]
2. Use validated *in vitro* tests for gene mutation and chromosome aberrations	→	*negative* Assume no genotoxic potential[b]
	→	*positive* Classify as genotoxic[b]
↓ *ambiguous result*[c] ↓		
3. Conduct a short-term *in vivo* test[d]	→ →	*negative* Assume no genotoxic potential Assess potential for non-genotoxic carcinogenicity (Figure 9.2)
↓ *positive result* ↓ Classify as genotoxic (somatic cell mutagen)	→	assume carcinogenic potential
↓		
4. Use a combination of a validated germ cell mutagenicity test and biokinetic data[e]	→	*negative* Not a germ-cell mutagen
↓ *positive or ambiguous result* ↓ Classify as genotoxic (germ cell mutagen)		

[a]*The computer-based prediction should be used in conjunction with* in vitro *data, according to a weight-of-evidence approach.*

[b]*In most cases, the assessment of genotoxic potential would stop here; the need for further genotoxicity testing depends on usage and exposure levels.*

[c]*An ambiguous result could be followed up by* in vivo *testing, or by returning to step 2 and conducting different* in vitro *tests.*

[d]*Usually bone-marrow micronucleus or metaphase analysis, and/or unscheduled DNA synthesis in liver; the need for the short-term* in vivo *test should be assessed on a case-by-case basis.*

[e]*In general, somatic cell mutagens are assumed to be potential germ cell mutagens; this step is therefore optional, to provide a means of assessing whether a somatic cell mutagen is, or is not, a germ cell mutagen.*

(Q)SAR(s) = (quantitative) structure–activity relationship(s).

genicity by way of a genotoxic mechanism. In this case, and depending on likely exposure levels in humans, a rodent bioassay may be required.

The detection of genotoxicity in different target tissues

The intraperitoneal route of administration is often used in short-term genotoxicity assays, supposedly to maximise the amount of compound entering the body, and to minimise the possibility that a negative result in the bone-marrow could have been caused by a lack of transport to this target site. It is also possible to assess systemic toxicity to the bone-marrow by assessing the ratios of normochromatic and polychromatic erythrocytes in the presence and absence of chemical exposure. A negative result in a bone-marrow micronucleus test, accompanied by evidence of distribution to the bone-marrow, is evidence of lack of genotoxicity. However, when there is no indication of transport to the bone-marrow, a negative result is impossible to interpret.

Other *in vivo* assays are sometimes required, especially if evidence from pharmacokinetic studies indicates that tissues other than the bone-marrow are targeted. Such assays include the detection of unscheduled DNA synthesis (UDS) in the liver, and the use of transgenic rodent systems, developed to detect mutagenicity simultaneously in a wide range of tissues. However, these adjunct tests are not used routinely, and have not been validated according to recognised international criteria (6).

In vivo genotoxicity tests are unsatisfactory for reasons other than the above, in that they are restricted to a few tissues, and they are limited to detecting effects caused by a narrow range of mechanisms, such as cytogenetic damage and DNA repair (except in the case of the relatively new transgenic rodent mutagenicity models, which still require formal validation).

The detection of aneupolidy

Changes in chromosomal number (polyploidy or aneuploidy) can be investigated both *in vitro* and *in vivo* by using metaphase analysis and chromosomal painting techniques, although these are labour-intensive methods. Changes in chromosome number will lead to alterations in the genotype, and are therefore considered to be genetic changes. However, aneuploidy can arise as a result of both genotoxic and non-genotoxic events (as defined in this chapter), since loss of chromosomes can be caused either by direct effects on the chromosome to produce an acentric fragment, or by interference with the site of attachment of the chromosome on the spindle.

Some authorities have called for the development and use of specific tests for aneuploidy (3). In relation to this, there have been efforts to develop an *in vitro* micronucleus test, although technical difficulties with this assay have delayed its validation, which is still in progress (14). The availability of an *in vitro* micronucleus test would be a considerable advance on the current situation, because scoring micronuclei is considerably easier than is scoring chromosomal damage, making the time required and cost of *in vitro* cytogenetics testing equivalent to those of the *in vivo* assay. Also, micronuclei can be formed by genotoxic and non-genotoxic events, with the possibility of separately quantifying the amount of each event (15, 16).

Current Approaches to Carcinogenicity Testing

The rodent bioassay

The conventional approach to carcinogenicity testing is the life-time rodent bioassay in rats and mice of both sexes, with full pathological analysis of all tissues (OECD TG 451). This test is time-consuming, labour-intensive and costly, both financially and in terms of animal numbers and animal welfare. The rodent bioassay is used to detect complete carcinogens, as well as tumour promoters and co-carcinogens (agents that can increase the frequency of tumorigenicity by acting in combination with another chemical or subsequent to another chemical). Moreover, rat and mouse data do not correlate well, and extrapolating the information to humans is problematical (17, 18). A scheme for carcinogenicity testing, based on genotoxicity assays and other tests, is presented in Figure 9.2.

The detection of genotoxic and non-genotoxic carcinogens

Results from the US National Toxicology Program (NTP) have shown that, in general, genotoxic chemicals are likely to exhibit trans-species carcinogenicity, often in both sexes, at intermediate dose levels, and this will not necessarily be restricted to one target tissue (19). These chemicals are the so-called "genotoxic carcinogens". The carcinogenicity of the vast majority of these chemicals could have been predicted by undertaking genotoxicity testing, without the need for the bioassay. However, an increasing number of chemicals lacking any genotoxicity are proving to exhibit carcinogenicity, and this activity is often manifested in one species, one sex and even in one specific tissue, and usually only at high dose levels (20). These chemicals are the so-called "non-genotoxic carcinogens".

The carcinogenic activity of non-genotoxic carcinogens cannot be predicted by the standard battery of genotoxicity assays (21). In contrast to genotoxic carcinogens, which act by damaging DNA, non-genotoxic carcinogens are known to act, or thought to act initially by a number of different mechanisms, some of them involving binding with specific intracellular receptors. At present, there are few *in vitro* methods for detecting non-genotoxic carcinogens, and none of them have been validated for regulatory usage. Therefore, chemicals that prove to be non-genotoxic *in vitro* and *in vivo* may still be subjected to a rodent bioassay, depending on their intended use, and if a high level of human exposure is anticipated.

In recent years, the relevance of non-genotoxic carcinogens to human hazard has been questioned, in view of their restricted activity profiles, especially since their effects are often detected exclusively in the mouse. As a consequence, there have been proposals by groups such as the International Conference on Harmonisation (ICH; 22) for mouse transgenic assays to be developed and validated as an alternative to the mouse bioassay (23). Some of these assays have been designed specifically to detect certain types of non-genotoxic carcinogen, whereas others are specific for chemicals with genotoxic activity. However, in a collaborative study organised by the International Life Sciences Institute (ILSI), no single transgenic mouse system or combination of systems has yet emerged that exhibits a sufficiently high sensitivity and specificity for rodent carcinogenicity to be considered relevant and reliable for regulatory use. In fact, the

results from the ILSI study raise several new questions: a) there appears to be an overlap between the activities of genotoxic and non-genotoxic chemicals in some assays, prompting questions regarding the different modes of action of these types of chemicals; b) the mechanisms by which enhanced tumorigenesis arises in some of the models in response to carcinogens are not fully understood; and c) more work needs to be undertaken before standardised and optimised protocols become available (24). It is also the case that any testing strategy involving the combined use of a rat bioassay with a mouse transgenic assay to detect carcinogens, still suffers from the need to undertake rodent-to-human extrapolation.

Modelling carcinogenesis *in vitro*

It is well established that the carcinogenic process can be modelled *in vitro* on the basis of morphological cell transformation (25). Several cell transformation assays based on immortalised or non-immortalised rodent cell systems were developed long ago, and have been shown to be capable of detecting some well-known animal and human carcinogens, as well as tumour promoters. In fact, many of the presumed stages of carcinogenesis, as well as the role of oncogenes, have been established as a result of *in vitro* studies on cell transformation.

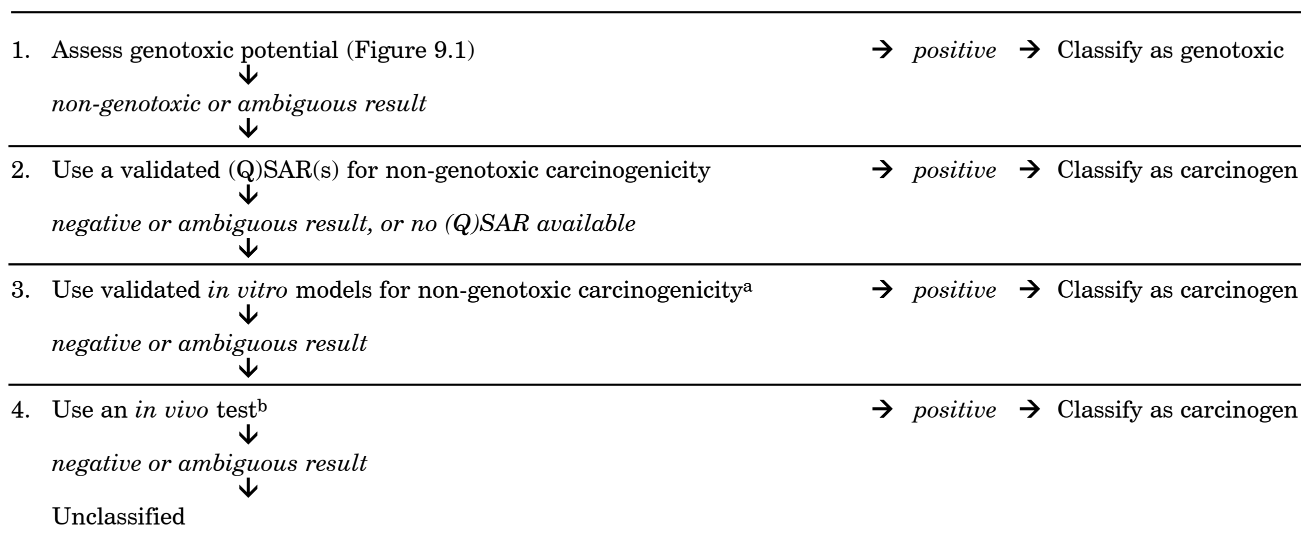
The main endpoints of cell transformation, focus formation and the acquisition of the ability to grow in soft agar, result from a loss of contact inhibition and loss of anchorage-dependence, respectively. The relationship between these cellular endpoints, which can be detected and quantified in cell culture and *in vivo* carcinogenesis and malignancy, can be demonstrated by malignant tumour formation after the transformed cells are injected into animals.

Modelling the later stages of carcinogenesis is increasingly difficult. Thus, *in vivo* experiments on cancer cell invasiveness and metastasis are limited in the detail that they provide, so it essential to implement *in vitro* studies. Good examples are the three-dimensional *in vitro* invasiveness assays of Mareel *et al.* (26), Smolle *et al.* (27), and Fusenig & Boukamp (28), as well as the refinement of the latter technique by DeVaney *et al.* (29). In addition, tumour neoangiogenesis can be studied in the allantoic membranes of chicken eggs.

Cell transformation assays

Recently, new protocols have been developed for two cell transformation systems, the Balb/c 3T3 and Syrian hamster embryo (SHE) cell assays, which have improved the reliability and predictivity of these assays for detecting rodent carcinogens (25, 30). These developments have also resulted in an increase in the database for the assays, and have provided evidence that the techniques can be performed in different laboratories with a high level of reproducibility. This is especially so in the case of the SHE cell assay, which exhibited a high level of sensitivity for rodent carcinogens in comparison with several other alternative assays to the conventional rodent bioassay — including transgenic

Figure 9.2: A tiered testing strategy for carcinogenicity



[a]*Cell transformation assay,* in vitro *micronucleus assay, aneuploidy assay.*

[b]*Rodent bioassay or transgenic mouse model; the need for the* in vivo *test should be assessed on a case-by-case basis.*

(Q)SAR(s) = (quantitative) structure–activity relationship(s).

mouse strains — in the recent ILSI collaborative study (31). In this study, the SHE cell assay was highly predictive of the rodent bioassay, and it also detected human carcinogens, but was poor at distinguishing between rodent and human carcinogens (32). It is noteworthy that ten out of 35 non-carcinogens were positive, though the mechanisms involved in generating these false-positives are unknown. The four non-genotoxic carcinogens in the study were positive when exposure was continued for seven days.

An analysis of a database containing results for 48 chemicals tested in the reduced-pH-protocol assay showed an 85% concordance between transformation and *in vivo* rodent carcinogenicity, with values for sensitivity and specificity of 87% and 83%, respectively (33). Since these reviews were published, sufficient additional data have become available under the US NTP collaborative study and additional studies, for an analysis of data on 75 chemicals tested at pH 6.7 to be undertaken. The results revealed an overall concordance of 83%, a sensitivity of 83% and a specificity of 82% (34).

Screening for non-genotoxic carcinogens

The ILSI study and other investigations have shown that some of the cell transformation assays are sensitive to a wide range of both genotoxic and non-genotoxic carcinogens, especially those that act by way of epigenetic effects and as tumour promoters, and might affect cellular proliferation. Also, a two-stage cell transformation assay in Balb/c 3T3 cells seems potentially useful for detecting tumour promoters (30). Morphological cell transformation has been shown to arise from point mutation, chromosomal damage, aneuploidy and other effects associated with cellular proliferation. However, because of the wide range of mechanisms by which non-genotoxic carcinogens might act, and especially as some of the effects are highly tissue-specific (for example, thyroid carcinogenesis and peroxisome proliferation), it is unlikely that a combination of this assay and one for aneuploidigenic agents would be sufficient to detect all types of non-genotoxic carcinogens.

Therefore, to increase the spectrum of non-genotoxic carcinogens that can be detected *in vitro*, it will be necessary to develop a battery of assays that involve the detection of the principal endpoints by which these agents act. For example, there are several cell culture methods for investigating the tissue-specific induction of proliferation, such as an MCF-7 cell assay, which are being used to detect hormonally-active chemicals and the links with breast cancer (35). In addition, several of the receptors for non-genotoxic hepatocarcinogenesis have been identified, which will facilitate the development of rapid ligand-binding assays (21).

The regulatory status of cell transformation assays

There are several reasons why regulatory agencies have been reluctant to adopt cell transformation assays for routine testing. These include: a) the perception that the assays are technically demanding; b) the variability of results among different laboratories; c) uncertainties about data interpretation, and scepticism about the direct relationship between the cellular endpoint and tumour formation *in vivo*; d) a lack of information regarding the predictivity of some of the assays for rodent and human carcinogenesis; and e) the lack of formal validation of any of the cell transformation systems. It is mainly for these reasons that cell transformation has remained a tool for research on mechanisms of action of chemicals on a case-by-case basis, rather than becoming a regulatory approach for general application.

The problem regarding the demanding nature of the cell transformation assays needs to be addressed as a matter of urgency. Thus, although the low-pH protocol for the SHE cell assay can be performed in different laboratories, it is considerably more complicated than the protocols required for other rodent cell transformation assays. Thus, it is suggested that all cell transformation assays should be developed further before being evaluated for their usefulness in predicting carcinogenicity.

Few regulatory authorities accept cell transformation data for anything more than providing mechanistic information. For example, the US Federal Drug Administration (FDA) indicates that cell transformation data can aid in compound selection and can give useful mechanistic information, even in the case of negative results. Clearly, there is an important need for regulatory bodies to assess the current status of rodent cell-transformation assays and their suitability for the detection and characterisation of carcinogens.

Human cells have been shown to undergo morphological transformation in tissue culture, and there is the possibility that reliable and sensitive human cell-based transformation assays could be developed soon. Clearly, the eventual development of a human cell transformation assay would obviate the need for inter-species extrapolation (25).

SARs for predicting genotoxicity and carcinogenicity

Considerable advances are being made in predicting genotoxicity and carcinogenicity by developing and analysing databases, and by applying computer-based approaches. Diverse structural alerting features of genotoxic compounds have been recognised, particularly from the results of the US NTP collaborative study on short-term tests for car-

cinogenicity, and these have been used in predicting carcinogenicity, especially by genotoxic mechanisms (36). By using fragment analysis, some 18 different structural alerts (toxicophores) have been identified as being present in chemicals that had been shown to be rodent carcinogens in two-year rodent life-time bioassays, as well as possessing genotoxicity in one or more short-term genotoxicity assays. However, fragment-analysis approaches pay insufficient attention to the effect that one substituent group may exert on another, and there will be an increasing need for more-sophisticated methods, such as QSAR methods and three-dimensional modelling, coupled with knowledge-based expert systems (37).

Expert systems

The computer-based expert systems for predicting the genotoxicity and/or carcinogenicity of chemicals are based on a variety of techniques, but essentially are divisible into automated rule-induction (ARI) and knowledge-based (KB) systems (38). These systems differ fundamentally in the way they operate, in that ARI systems predict by learning from and discovering patterns in existing data, whereas KB systems predict by reasoning on the basis of existing human knowledge. ARI systems make quantitative predictions (for example, by providing a probability value that carcinogenicity will be induced by a molecule).

Three widely used ARI systems are TOPKAT (Toxicity Prediction by Computer-assisted Technology), CASE (Computer Automated Structure Evaluation) and COMPACT (Computerised Optimised Parametric Analysis of Chemical Toxicity). Examples of KB systems are HazardExpert and DEREK (Deductive Estimation of Risk from Existing Knowledge), both of which are used to predict a wide variety of toxicity endpoints, and ONCOLOGIC, which is based on a decision-tree approach. There are also some KB systems for predicting metabolism, one example of which is a new program called METEOR.

ARI systems analyse information on structures entered into the computer for associations between inactive and active molecules. Molecules are fragmented into all possible atom pairs and other associations. Then, pattern-recognition and cluster-analysis techniques are used, together with statistical analyses, to compare the frequencies of occurrence of specific structural features in sets of active and inactive molecules, for a particular biological endpoint, by using a training set of chemicals. In this way, the most important features determining or modifying activity are identified. Such features are then searched for in novel molecules, which are entered into the trained system, and which are then examined for the presence of biophores and biophobes. Some ARI systems also use QSAR and molecular modelling of three-dimensional structure. There have been many publications showing how programs such as TOPKAT and CASE can predict the genotoxic and carcinogenic activities of chemicals (see, for example, 39).

COMPACT differs from these programs in that it analyses the ability of a molecule to fit into the active site of CYP1A1 (and some other CYP isozymes), by modelling molecular shape (planarity or area/depth) and chemical reactivity (covalent bond formation). The use of COMPACT is, of course, limited to molecules that are activated by these CYP enzymes.

DEREK is now one of the most widely used KB systems, and has an extensive rule-base for genotoxicity, and especially for mutagenicity, initially based on the so-called Ashby alerts (see also, Chapter 6). However, this rulebase has been added to extensively and refined, especially through feedback from the DEREK-users group (40). The rulebase for carcinogenicity has also been continually refined, particularly by the recent rewriting of the FDA rulebase that applies structural alerts to all epoxide substructures. Some mutagenicity and carcinogenicity rules have also been written for chemicals found in foods (41).

The validation of expert systems for genotoxicity and carcinogenicity

There have been few studies in which expert systems have been compared for their ability to correctly predict the same biological activity, except in the case of rodent carcinogenicity, by using the NTP database (42–45). In these studies, several of the systems discussed above showed overall accuracies in correctly identifying rodent carcinogens, which varied from 60% to 90%, depending on the system and the database. Optimal levels of performance were obtained by using combinations of the systems. It can be concluded from these kinds of studies that expert systems should be used as screens in conjunction with each other and with *in vitro* tests. However, it is most important that expert systems should be validated according to the principles and procedures applied to other test methods. Unfortunately, this has not yet happened and, in some situations, systems such as COMPACT and CASE are apparently used by only one research group.

Expert systems are developed by using training sets of chemicals. It is important that such training sets contain chemicals with different structures and with a range of biological activities. Training sets should also contain chemicals that act to cause the toxic effects of interest through as wide as possible a range of mechanisms. It is crucial that systems are developed that can correctly predict not only the

activities of the chemicals in the training set, but also those of different chemicals, which are structurally related to those in the training set. This is so that the utility of the expert system for predicting the activities of novel chemicals is as wide as possible.

The limitations of expert systems

Expert systems have several important limitations. Firstly, their development depends crucially on the availability of accurate and relevant biological data of high quality on individual chemical entities with well-defined structures. Unfortunately, no test sample is completely pure, and it is important that information used to construct the rules for expert systems is derived from studies on test samples of high purity. Thus, strict criteria should be applied to the process of data acceptance for rule development, and ideally, the effects of metabolism should be known, or should be predictable.

Structure-activity relationships for non-genotoxic carcinogens

The mechanisms involved in non-genotoxic carcinogenesis are less well-characterised than are those for genotoxic carcinogenesis. As a consequence, considerably less information is available for generating useful rules for predicting this type of activity. This issue was discussed by Combes (36), who also gave examples of the identification of some structural features of molecules that might be associated with non-genotoxic carcinogenesis, including information on some hormonally active chemicals (21).

Tiered Testing Strategies for Genotoxicity and Carcinogenicity

The principal problem in the development of a strategy for the mutagenicity and carcinogenicity testing of existing and new chemical entities revolves around two main questions: a) whether it is necessary to use *in vivo* genotoxicity assays; and b) whether it is necessary to test chemicals for carcinogenicity in the rodent bioassay or an alternative (equivalent) assay in animals.

It can be argued that, if a chemical is genotoxic *in vitro*, it should be regarded as a genotoxin with the potential to induce germ-line mutations and carcinogenicity, irrespective of its intended usage. The justification for using *in vivo* tests is that the chemical could prove to be negative *in vivo*, because of detoxification (a process less likely to occur *in vitro*), transport away from the site of action, and exposure to more-realistic, lower dose levels. However, *in vivo* genotoxicity assays are unsatisfactory for other reasons (see above). Moreover, chemicals that are intrinsically genotoxic (without the need for exogenous metabolic activation), could exert effects in tissues after administration at initial sites of contact, before reaching the liver. In addition, chemicals requiring activation *in vitro* could be converted into reactive forms by extrahepatic metabolism in such tissues (10).

These arguments support the view that *in vivo* genotoxicity assays provide data of dubious relevance, and that the careful use and interpretation of data from a battery of *in vitro* tests might be a better approach. However, it is the usual experience in industry that a significant proportion of chemicals exhibit genotoxicity *in vitro,* but prove inactive *in vivo* after thorough testing. In other words, potentially useful and important chemicals could often be discarded unnecessarily, if only *in vitro* test data were used for regulatory purposes. This conclusion is based on the assumption that the relative insensitivity of *in vivo* assays reflects a lack of realisation of genotoxic potential in the whole animal. However, the scientific basis of the insensitivity of *in vivo* genotoxicity assays is not known.

It is therefore suggested that the need for further *in vivo* genotoxicity testing should be justified on a case-by-case basis, in relation to the stage of screening being undertaken, the justification for undertaking *in vivo* assays in relation to known target organ distribution, and the expected level of human exposure to the chemical.

A chemical that is negative *in vitro*, in at least a point-mutation assay and a cytogenetics assay, should be tested in a further *in vitro* assay, which should include aneuploidy as an endpoint, since this is known to be an important pathway for the effects of several chemicals that are uniquely genotoxic *in vivo* (3, 46–48). A positive result in such an assay should be considered as indicative of carcinogenicity through a non-genotoxic mechanism, and other evidence for this should be sought from conventional chronic and sub-chronic study data (see below), and from the use of other approaches (see below). Also, an ability to induce aneuploidy, coupled with evidence for transport to the germ cells, indicates the possibility of important germ–line effects, in which case the chemical should either be banned or be subjected to marketing restrictions. It should also be a general rule that chemicals that are unequivocally genotoxic (after testing *in vitro* and *in vivo,* as appropriate) should not be subjected to carcinogenicity bioassays. Tiered approaches for the assessment of genotoxicity and carcinogenicity are outlined in Figures 9.1 and 9.2, respectively.

Issues Requiring Further Consideration

The standard battery of *in vitro* and *in vivo* genotoxicity assays should detect all genotoxic carcino-

gens, but they will fail to detect most non-genotoxic carcinogens. One way in which the latter class of carcinogen might be detected is by the use of a cell transformation assay. It is, however, unrealistic at the present time to expect the regulatory authorities to endorse the use of transformation for regulatory purposes without the formal validation of optimised test protocols. It is therefore a matter of priority that the newly developed protocols for the Balb/c 3T3 and SHE cell transformation assays should be subjected to formal validation.

At the same time, there needs to be an ECVAM workshop to discuss the whole topic of non-genotoxic carcinogenesis, and to include detailed discussions on: a) the known modes of action of non- genotoxic carcinogens; b) the available and required non-animal testing strategies for detecting and characterising them; and, most importantly, c) their relevance to human hazard. The last-named discussion should focus on the evidence supporting the human carcinogenicity of non-genotoxic carcinogens, and how specific effects in rodents can be extrapolated to human hazard. Several non-animal methods are available, ranging from (Q)SAR studies and *in vitro* proliferation assays to aneuploidy assays.

A further perceived need for using the rodent bioassay or an equivalent assay for detecting non-genotoxic carcinogens is the possibility that these chemicals exhibit a threshold dose, in contrast to genotoxic carcinogens (49). The data from bioassays are therefore used for regulatory purposes, to set exposure limits below which carcinogenic risk is considered to be much reduced. Most regulators consider that dose–response data from *in vitro* assays cannot be used to establish threshold doses, and use this argument to justify the use of *in vivo* experiments for this purpose. However, the current approach could be replaced in the following ways: a) by evaluating whether certain classes of non-genotoxic carcinogens, such as peroxisome proliferators, are relevant to human hazard; and b) by the combined use of information from toxicokinetic and chronic toxicity studies, in which target organ doses can be established (50) in relation to early endpoints of non-genotoxic carcinogenesis, such as cell proliferation (51).

Some regulatory agencies have, in fact, explored the possibility of using dose–response data from certain *in vitro* assays (for example, *in vitro* aneuploidy) for risk-assessment purposes, and this issue would also need to be explored in the proposed ECVAM workshop.

There are two further issues with regard to genotoxicity and carcinogenicity testing: a) the focus by industry and regulatory bodies on predicting rodent carcinogenicity; and b) the unrealistic expectations being raised regarding the advantages of approaches based on genomic and proteomics.

In the first case, discussions with toxicologists in industry have confirmed the view that there is a disproportionate emphasis on finding alternatives to *rodent* bioassays, since this is the conventional route in regulatory toxicology. This approach is erroneous, since, ultimately, we should be interested in *human* carcinogens, and the relevance of rodent bioassay data to humans is questionable. This situation prompts the use of rodent *in vivo* data for validation studies on alternative methods, with insufficient use of the available human and mechanistic data.

In the second case, it is becoming increasingly clear from the outcome of several recent conferences and workshops, as well as from the literature, that expectations of genomics and proteomics have far exceeded the ability of these approaches to provide useful and reliable data that can be interpreted in terms of standard toxicity endpoints (52, 53). This has largely been because of the way in which the sensitive molecular techniques have been applied. As a result, there have been projections that it will be at least 10 years before any useful information can be derived from genomics and proteomics. This needs to be recognised and acknowledged by the regulatory authorities and funding bodies, so that efforts to develop more-conventional *in vitro* toxicity methods and tests are not reduced, in the expectation that genomics and proteomics will soon provide all the answers.

Genotoxicity and Carcinogenicity: Summary, Conclusions and Recommendations

QSAR, expert system and *in vitro* approaches could be used immediately to prioritise chemicals for further testing on the basis of their potential genotoxicity and carcinogenicity. For the definitive assessment of genotoxicity, negative results in a bacterial assay for gene mutation and in a mammalian assay for clastogenicity/aneugenicity would normally be regarded as sufficient evidence for lack of genotoxic potential.

For carcinogenicity testing, the rodent bioassay is not suitable for dealing with large numbers of chemicals, not only because of the considerable time and cost involved, but also because of the large numbers of animals required. Therefore, it is important that alternative methods for the definitive assessment of carcinogenicity are further developed and subjected to validation.

Short-term prospects

1. A review of the current validation and regulatory status of aneuploidy and micronucleus assays in mammalian cells, to include an assessment of the range of aneugens detected by both methods, with a view to eliminating redundancy.

2. A review of the current validation and regulatory status of rodent cell transformation assays (especially the SHE cell and Balb/c 3T3 systems), and appropriate action to facilitate the validation and acceptance of one or both of these systems.

3. A review of the need to conduct *in vivo* genotoxicity testing, especially for chemicals that are not intended for human consumption or direct exposure, and those for which indirect exposure is expected to be negligible.

4. The development of receptor-binding assays for non-genotoxic carcinogens.

5. The further development of QSAR and other computer-based approaches for mutagenicity and carcinogenicity.

Medium-term prospects

1. An appraisal of the usefulness of genomics and proteomics in the development of *in vitro* methods for genotoxicity testing.

2. The validation of receptor-binding assays for non-genotoxic carcinogens.

3. The validation of QSAR and other computer-based approaches for predicting genotoxicity and carcinogenicity.

Recommendations for research and development

1. Further research concerning: a) the known modes of action of non-genotoxic carcinogens; b) their relevance to human hazard; and c) the available and required non-animal testing strategies for detecting and characterising those carcinogens that are relevant.

2. The development of human cell-based cell transformation assays.

Other recommendations

1. Less emphasis should be placed on the development and validation of transgenic rodent models of carcinogenesis as assays for detecting carcinogens, since these are unlikely to be capable of identifying rodent and/or human carcinogens.

2. A way needs to be found, perhaps through the ICH process, and through relevant regulatory agencies responsible for non-pharmaceutical products and chemicals, to change the emphasis from predicting rodent carcinogenicity to predicting potential human carcinogenicity.

3. ECVAM should organise workshops on:

   a) non-genotoxic carcinogens, in conjunction with the FRAME Toxicity Committee and the International Agency for Research on Cancer (IARC), to discuss and make recommendations for: i) the known modes of action of non-genotoxic carcinogens; ii) their relevance to human hazard; and iii) the available and required non-animal testing strategies for detecting and characterising those carcinogens that are relevant; and

   b) the use of genetically engineered cell lines for predicting metabolism-mediated genotoxicity.

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