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Genetic Toxicology*

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Abstract

Systems for testing genetic toxicology are components of carcinogenic and genetic risk assessment. Present routine genotoxicity-testing is based on at least 20 years of development during which many different test systems have been introduced and used. Today, it is clear that no single test is capable of detecting all genotoxic agents. Therefore, the usual approach is to perform a standard battery of in-vitro and in-vivo tests for genotoxicity. Work-groups of the European Union (EU), the Organization for Economic Co-operation and Development (OECD), and, very recently, the work-group of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) have defined such standard battery tests. These and some currently used supplementary or confirmatory tests are briefly discussed here.

Additional test systems for the assessment of genotoxic and carcinogenic hazard and risk are seriously needed. These tests must be more relevant to man than are current assays and less demanding in respect of cost, time and number of animals. Another aspect for re-assessment derives from the actual situation in the pharmaceutical industry. Companies have to prepare for the world economy of the 21st century. Therefore, pharmaceutical research is speeding up tremendously by use of tools such as genomics, combinatorial chemistry, high throughput screening and proteomics. Toxicology and genotoxicology need to re-evaluate their changing environment and must find ways to respond to these needs.

In conclusion, genetic toxicology needs to answer questions coming from two major directions: hazard and risk identification and high throughput testing.

Genetic toxicology is the attempt to provide information essential to maintaining the integrity of our genetic material to prevent or slow down cancer, hereditary disease and ageing. Genetic toxicology studies damage to genetic material (i.e. genes, DNA) by chemical or physical agents. Damage to the genetic material, if not repaired rapidly and correctly, changes the DNA sequence. If the changes are not lethal they will lead to heritable changes, i.e. mutations.

DNA sequence changes can be single nucleotide changes that result in point mutations, or multiple nucleotide changes that result in visible chromosomal aberrations. Mutations often result in the elimination or alteration of gene function. The adverse effect of a mutation depends on the gene, the site in the gene, and the tissue affected. The most serious effects of mutations in germ cells are birth defects, and neoplasms in somatic cells.

The somatic cell mutation theory of carcinogenesis, introduced almost 70 years ago (Boveri 1929) postulates that cancer can be caused by mutations. Recent developments in molecular cancer genetics show that carcinogenesis is often associated with mutations in oncogenes and anti-oncogenes (tumour-suppressor genes). In fact, multiple mutations and genetic alterations have been demonstrated to be necessary for development of tumours in man. The association of mutations with neoplasms supports the somatic cell mutation theory. Accordingly, it still seems reasonable to use tests on genotoxicity in somatic cells or in in-vitro test systems to identify carcinogens.

Compounds which produce positive results in such tests have the potential to be carcinogens or mutagens in man, i.e. they might induce cancer or heritable defects. Although the relationship between exposure to particular chemicals and carcinogenesis has been established for man, a similar relationship has been difficult to prove for inherited diseases. For this reason, genotoxicity tests have

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been used mainly for prediction of carcinogenicity. Nevertheless, because germ line mutations are clearly associated with disease in man, the suspicion that a compound might induce heritable effects is considered to be at least as serious as the suspicion that a compound might induce cancer. In addition, the outcome of such tests might be valuable for the interpretation of carcinogenicity studies.

Present State of Genotoxicity Testing

The tests in current use go back to the science of the early 1970s. During more than 20 years of routine genotoxicity testing many different test systems have been introduced and used. Today it is clear that no single test is capable of detecting all relevant genotoxic agents. Therefore, the usual approach is to perform a battery of in-vitro and in-vivo tests for genotoxicity. Work-groups of the EU, the OECD, and, very recently, the work-group of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH 1997) have introduced such a battery of tests. Such international harmonization has led to the concept of a standard battery of tests to be conducted for each compound and additional confirmatory or explanatory tests depending on the results of the standard battery and of structural alerts. The following battery of standard tests has been recommended by the ICH work-group.

Bacterial reverse-mutation test

This test, developed by Bruce Ames and his colleagues in the early seventies (Ames et al 1975), is extensively used for testing the mutagenicity of chemicals. It is based on a set of genetically altered histidine-requiring strains of the bacterium *Salmonella typhimurium*. It is called a reverse-mutation assay because the bacterial strains used are mutated in one of the genes responsible for histidine biosynthesis and consequently cannot synthesize histidine, an essential amino acid. An additional mutation, e.g. caused by a mutagenic chemical, is required to revert the cells to histidine-independence. In common with the other in-vitro assays, this system also requires the use of an exogenous source of metabolic activation.

In-vitro test for chromosomal damage—the in-vitro mouse lymphoma thymidine kinase assay

These systems, which identify substances that cause chromosome aberrations in cultured mammalian cells, employ cultures of established cell lines or primary cell cultures and exogenous metabolic activation (Preston et al 1981).

In the cytogenetic assay, cell cultures are exposed to the test substance both with and without metabolic activation. Lymphocytes from man or cells from standard cell lines are in routine use. At predetermined intervals after exposure they are treated with a metaphase-arresting agent (colchicine), harvested and stained. Metaphase cells are analysed microscopically for the presence of chromosome aberrations.

In the mouse lymphoma thymidine kinase assay, L5178Y mouse lymphoma cells are exposed to the test substance both with and without metabolic activation. The genetic endpoint in this cell line measures mutations at the thymidine kinase gene locus. Thymidine kinase is a key enzyme for biosynthesis of DNA nucleotides and catalyses the formation of the pyrimidine nucleotide thymidine triphosphate from thymidine monophosphate. Cells deficient in thymidine kinase activity are resistant to the cytotoxic effect of the pyrimidine analogue trifluorothymidine (TFT) because they cannot use it. Thymidine kinase-proficient cells are sensitive to TFT, which causes inhibition of cellular metabolism and halts further division. Thus mutant cells proliferate in the presence of TFT, whereas normal cells, which contain thymidine kinase, do not. The molecular dissection of mutants induced at the thymidine kinase locus shows a broad range of genetic events including point mutations and chromosomal aberrations such as deletions, translocations and recombinations, etc.

However, recently published results (Storer et al 1997) indicate that tumour-suppressor protein p53 is dysfunctional in L5178Y cells. It is therefore suggested that this cell line, because of delayed apoptosis, increased recombination, and survival at higher doses, might be abnormally susceptible to the induction of genetic alterations (mutations).

In-vivo test for chromosomal damage

The micronucleus test is the most widely used in-vivo test for chromosomal damage in which polychromatic (immature) erythrocytes from bone marrow are the target cells (Salamone et al 1980; Preston et al 1981). Cytogenetic damage can result in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes. The first kind occurs if a chromosome is not at the right place in mitosis or if chromosome distribution is not correct; these micronuclei appear soon after treatment with the substance. The second kind requires chromosome breakage and usually appears much later after treatment. When a bone marrow erythroblast develops into a polychromatic erythrocyte, the main nucleus is extruded. Any micronucleus that has been formed might remain

behind in the otherwise unnucleated cytoplasm. An increase in the frequency of micronucleated polychromatic erythrocytes in treated animals is an indication of numerical or structural chromosome damage.

The overall risk assessment process can be divided into several steps: hazard identification, dose response characterization, exposure characterization, risk assessment, and risk management. The first step of this process, hazard identification, is the qualitative scientific evaluation of the adverse effects of a substance on man or other organisms. It usually comprises in-vitro and in-vivo toxicity data, pharmacokinetic data and consideration of structure–activity relationships and types and extent of use. Dose–response characterization, exposure characterization, and finally risk assessment are further steps that are also part of a scientific process based on data and extrapolations. Risk management is mainly a political process based on opinions.

The basic idea is that a test battery such as the ICH standard test battery should enable a hazard identification in respect of damage to DNA and its fixation. The problem is that principally in-vitro genotoxicity tests might identify the genotoxic potential of a compound but they do not identify a real hazard. By adding in-vivo tests it would be possible to come closer to the goal of genotoxic hazard identification. However, it can easily be understood that the addition of a single cytogenetic in-vivo test to the standard test battery, using extremely high doses and a single administration, might not be sufficient.

Therefore the ICH work-group, in common with other scientific groups, demands additional tests to be conducted to ensure hazard identification is sufficiently reliable. These are tests for measurements of DNA adducts, DNA strand-breaks and DNA repair or recombination. However, even if these tests led to clear identification of genotoxic substances, they would not enable quantification of risk to man. But it is neither practical nor necessarily advisable to eliminate exposure of man to all substances that have been identified to be genotoxic. What is needed is information that can be used to reduce uncertainties in extrapolating risk to man from experimental data. In this respect it is especially important to have experimental systems with which to evaluate the relevance of high-dose effects found in routine assays to the effects of low doses and to have experimental test systems that are predictive of a particular outcome in man.

For genotoxic risk evaluation it is regarded as necessary to study the effects of potential mutagenic agents in germ cells. The mouse specific-locus test was developed for this purpose (Russel 1951). To this day, the mouse specific-locus test has served as a statutory standard for the assessment of heritable damage. However, thousands of animals are used for each test and treatment is usually performed with high doses of a potential mutagen.

The statutory standard for assessing the carcinogenic risk of a chemical in-vivo is the long-term rodent carcinogenicity bioassay. In this bioassay, rodents are treated with the maximum tolerated dose (MTD) throughout their lifetime and monitored for the occurrence of tumours. This study is expensive, time-consuming (3–4 years), requires a relatively high numbers of animals (approx. 500) and is often conducted at near toxic doses, which do not reflect the low-dose ranges to which man is exposed.

New Perspectives in Genotoxicity

After two decades of development, refinement and validation, the tests described above are well-established in routine testing schemes such as the ICH standard test battery, but our views of their utility for safety evaluation have to be re-assessed. The genetic targets of the 1970s were usually unknown and were chosen for experimental convenience. It has become clear, though, that other test systems for the assessment of genotoxic and carcinogenic hazard and risk are seriously needed. Today there are many new technologies that could be used. We now know substantially more about the selectivity of DNA–chemical interactions and the importance of critical sequences in DNA (e.g. in the p53 gene) the alteration of which might be an essential element in some cancer-inducing processes (Elespuru 1996). New tests must be more relevant for the situation in man than the present assays and should be less demanding in respect of cost, time or the number of animals used. They must meet requirements regarding: extrapolation from high dose to low dose; mechanistic connection to health outcome (e.g. p53 mutation and cancer development); and simple, reliable technology that could easily be integrated into existing toxicology protocols and universal validation.

Re-assessment is also necessary because of the actual situation in the pharmaceutical industry. Because companies and governments have to prepare for the world economy of the 21st century, toxicologists, genotoxicologists and other scientists need to re-evaluate their changing environment (Watanabe 1997). Modern pharmaceutical research is speeding up tremendously by use of tools such as genomics, high throughput screening and proteomics. Toxicology and genotoxicity must find

ways to respond to these needs. In effect the field of genotoxicity testing is facing pressure mainly from two directions: hazard and risk identification, and high throughput testing.

Hazard and Risk Identification

Not all toxic hazards act by the same type of pathological mechanism (Vainio 1995). On the basis of the mechanism of disease causation and the reversibility of effects, toxic effects can be divided into two main classes: traditional deterministic toxicity (Paracelsus) and stochastic toxicity. Deterministic toxicity overwhelms the body's compensatory processes, thereby exceeding the toxicity threshold and leading to pathological effects. Effects are reversible up to the late stages of the disease process. Every compound possesses the potential to produce toxic effects in this way. Toxic effects that arise from stochastic processes have no threshold for toxicity, so that exposure to low doses of an agent can lead to serious effects that are not necessarily related to the level of exposure. Furthermore stochastic effects are irreversible or poorly reversible. Not every compound has the potential to produce toxic effects in this way, the potential has to be identified experimentally. Genotoxic and carcinogenic effects are typical stochastic processes.

The reason for the success of in-vitro test systems in genetic toxicology is based on the ability of these test systems to identify the genotoxic potential of chemicals. However, they are not able to identify the genotoxic hazard of these chemicals to man. To extend the possibilities of genetic toxicology further in the direction of identification of genotoxic hazard and even to enable some risk evaluation, various approaches have been developed.

Metabolic activation

Many mutagens and carcinogens are not toxic as such but acquire biological reactivity after oxidation by certain cytochrome P450 enzymes. Cytochrome P450 enzymes are polymorphic enzymes that metabolize many endogenous compounds, including steroids, fatty acids and prostaglandins, and exogenous compounds, such as drugs and environmental contaminants. The specific cytochromes involved in detoxification or activation depend on the physicochemical characteristics of the chemical. Not only every species but every individual has a personal cytochrome P450 profile, and the activation and inactivation of toxic chemicals vary from one individual to another. Therefore the aim of research in this field is to identify the contribution of each P450 enzyme to the activation of a chemical. Assuming that species and individual P450 profiles could be determined, one could predict the ability of individuals to expose themselves to active metabolites. Although science has not yet reached that stage, it might indeed be possible in the not too distant future to diagnose and predict individual metabolic capacity on the basis of analyses of coding in DNA and RNA for cytochrome P450 enzymes and other genetically determined polymorphisms in the conjugation enzymes, such as glutathione *S*-transferases and *N*-acetyltransferases.

Presently, genetic toxicology often uses mice as test animals or in-vitro metabolic systems, both far from being identical to the situation in man. The most widely used in-vitro metabolism system is the rat-liver microsomal system. It is commonly performed using the S9 fraction, which contains many of the enzymes present in-vivo. The impressive success of this system also comes from the variety of genetic test systems with which it can be combined. Critical features are: lipid peroxidation, proteolytic activity, thermal denaturation, haem inactivation by reactive intermediates (suicide inactivation of CYP), non-specific binding (toward non-microsomal proteins) and spontaneous decay of the active intermediate.

Molecular biology

Molecular approaches (Vainio 1995) provide the means of studying underlying mechanisms and can be used to identify causal associations, that would otherwise be obscure, and to make better quantitative estimates of those associations at relevant levels of exposure. The use of molecular approaches in toxicology should, thus, result in better estimates of risks to health in man.

Macromolecular adducts. Risk assessment is ultimately based on quantitative estimate of the dose of the toxicologically relevant metabolites delivered to the target tissues. In the past such data were derived only by a lengthy process involving estimation of systemic doses in animals and man. A new form of dosimetry that is increasingly being used is based on analysis of the products of chemicals with macromolecules in-vivo. DNA adducts are considered to be a necessary but not sufficient condition for the initiation of chemical carcinogenesis or mutagenesis. Protein adducts might serve as surrogates or supplements for DNA binding.

DNA adducts. Several methods are currently available for detecting DNA adducts like O^6-methylguanine in exposed animals or man. These

include synchronous fluorescence spectrophotometry, the use of monoclonal antibodies and ^{32}P post-labelling. DNA adducts can serve as a measure of the biologically effective dose and thereby take into account inter-species and inter-individual variation in pharmacokinetic parameters. Some carcinogens do not generate DNA adducts. Some DNA adducts (e.g. O^6-methylguanine) seem to have biological significance whereas others (e.g. N^7-methylguanine) might not.

Methods that involve monoclonal antibodies offer the advantage of greater specificity than other methods of detection. Their major problems are still cross-reactivities of the antibodies used, with a number of adducts formed by structurally closely related chemicals.

The ^{32}P post-labelling method is extraordinarily sensitive and enables detection of one adduct in 10^{10} normal nucleotides. It is not specific however, in the sense that it only picks up most bulky DNA adducts and is only semiquantitative.

In experimental animals DNA adducts can be determined in almost all tissues. Lymphocytes are a readily available source of cells from man and are sometimes good surrogates. Another available source cells from man are exfoliated urothelial cells.

Protein adducts. There is increasing acceptance that haemoglobin adducts, for example, are a relevant indicator of the exposure of animals and man. Stable adducts remain available throughout the life span of erythrocytes (120 days). An assay of haemoglobin adducts gives a better estimate of past exposure than measurement of metabolites in blood or urine, which indicates only very recent exposure. The advantages of protein adducts as indicators are easy access, long biological half-life, dose-dependent covalent binding, and independence of inter-individual variations in intake, absorption and metabolism.

Receptor interactions

Current theories of chemical carcinogenesis implicate multiple and sequential somatic mutation and cell proliferation in neoplastic development. Many carcinogens, however, do not directly interact with DNA. Research during the past few years has revealed the existence of a class of genes that encode ligand-dependent DNA-binding proteins, called the steroid/thyroid receptor superfamily because the receptors belong to that class of protein. The aberrant forms compete with normal receptors for binding to the responsive element on DNA and, thus, interfere with the normal transcription control mechanism.

The selective action of non-genotoxic carcinogens (epigenetic) might be because of both cytotoxicity and receptor-mediated cell proliferation. Cytotoxicity is probably an effect of high doses, whereas receptor-mediated cell proliferation can occur at either high or low doses. An example of receptor-mediated tumour promotion is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which interacts with an intracellular protein (Ah receptor).

Cell-surface markers

Cell-surface markers such as glycophorin A, HLA-A and T-cell receptors are also studied because of their potential use as biomarkers for genetic toxicity testing, especially with regard to carcinogenicity (MacGregor 1997).

Oncogene products

Critical DNA sequences that are related to cancer development occur in a number of oncogenes and tumour-suppressor genes. Products of cellular oncogenes and anti-oncogenes are useful as biomarkers of events related to cancer risk. The oncogenes function at cellular levels encoding proteins that affect cellular life cycles and control basic processes related to cell division, growth, differentiation and apoptosis (programmed cell death).

A good example is the p53 tumour suppressor protein which plays an important role in regulating the cellular response to DNA damage, including cell cycle arrest and apoptosis induction. Both p53 functions are aimed at the maintenance of genomic stability (Storer et al 1997). The bcl2 gene product acts to prevent apoptosis, apoptosis being a key event with regard to cell survival after genotoxic stress (Kirsch-Volders et al 1997).

Molecular genetic analysis and cytogenetics

Substantial technological development has occurred in the area of genetic analysis and cytogenetics. DNA sequencing, DNA hybridization, and PCR (polymerase chain reaction) technologies now enable the routine analysis of mutants to discern the mutations they contain.

A good example of the use of such advanced techniques is the restriction-site mutation assay (Parry 1997). This test uses p53 intron sequences and can be performed with somatic or germ cell tissues. Genomic DNA is digested with restriction enzymes (e.g. EcoR1). Restricted DNA is analysed by PCR and electrophoresis. The test detects mutations and gives information about the hot spots

in an organism which could be used for screening purposes.

Another good example of modern molecular genetic analysis is the in-vitro or in-vivo single-cell gel assay (comet assay). With this test DNA strand-breaks can be detected in a small number of cells from a range of cell-types and tissues, e.g. skin (keratinocytes), nasal (respiratory/olfactory) tissue and germ cells (spermatocytes, oocytes) (Tice 1997). The sampled cells are lysed with alkali and electrophoresed through an agar gel. Cells containing DNA strand-breaks have the appearance of comets, with broken DNA streaming away from the body of the cell. Therefore the generic name for this technologically quite simple test system is comet assay.

Good examples of the development of molecular cytogenetic methods are fluorescence in-situ hybridization (FISH) and primed in-situ DNA synthesis (PRINS). These enable the identification of specific chromosomes, their centromeres and telomeres, and individual regions of interest in metaphase spreads and have thereby facilitated detailed analysis of chromosomal aberrations, including translocations or incorrect numbers of chromosomes, and have thus given some new insights into the mechanism of induction of chromosomal aberrations. The FISH technique can, for example, be used to detect aneuploidy in sperm cells (Adler & Wyrobeck 1997).

In future, the comet assay and the FISH technique might be combined by using specific DNA probes to detect DNA damage in selected sequences, regions or chromosomes.

Use of transgenic animals and cells

Birth defects (germ cell mutations), cancer, ageing and a variety of diseases have been shown to occur as a result of mutations in specific genes in specific tissues and cells. For example, cancer has been shown to be a result of mutations in oncogenes and tumour-suppressor genes. Until recently no reliable and practicable assay has been available. A revolutionary advance in this field has been the development of transgenic systems that enable easy quantitation of mutations in any tissue or cell from which DNA can be isolated.

This approach might greatly contribute to hazard identification and to risk assessment in genotoxicity and carcinogenicity. Transgenic animals will be constructed that enable uniquely sensitive in-vivo identification of carcinogens and mutagens and will provide insight into dosage and tissue specificity. They will also tell us more about the role of specific genetic alterations as predisposing factors in chemical toxicity.

The Transgenic Approach

The origins of genetic toxicology are firmly grounded in the concept of genetic engineering. As was mentioned in connection with the bacterial tests, Bruce Ames and his colleagues achieved one of the earliest applications of genetically-altered organisms in the field of genetic toxicology. Their *Salmonella* tester strains marked the start of systematic screening and routine testing for genotoxicity.

Of course, scientists had also recognized that the complexities involved in studies of mutagenesis across mammalian species could be reduced by using the same gene marker for mutation in a variety of species. A target for mutation analysis that is independent of any requirement for expression, growth or selection in tissue should be introduced into different host species. The capability to alter mammals genetically was, however, much more difficult to achieve.

Only within the last decade has it been possible to alter specific genes and then transmit them via the mammalian germ line. Once pronuclear injections and successful implants of blastocytes for embryo development had been accomplished, the methodology spread and evolved quite rapidly. Today it is possible to introduce almost any cloned gene into the germ line and study the expression pattern and effects of the introduced gene, or transgene. This has enabled the extension of in-vitro studies into whole-animal systems in which the introduced gene is subject to all normal regulatory processes from the onset of development. Transgenic animals are the primary model system for examining molecular genetic phenomena in-vivo.

The term transgenic has evolved to denote animals that have ectopic or altered genes. It is clearly an operational definition that is applied to a variety of animals. The resulting transgenic animals could be exposed and the transgene later recovered to observe mutations. Comparison of mutation rates could then be made between somatic tissues and correlated with other endpoints. Identical transgenes introduced into rodents or cultured cells from man would simplify the cross-species or cross-system comparison.

It is possible to group the various transgenic lines into three broad categories: transgenes altered in regulation and expression; non-expressed xenogenes (such as lambda lacI target or neutral lacZ reporter genes); and knock outs or genes whose function is ablated or altered through homologous recombination.

Thus far, hundreds of different transgenic mouse

lines have been created and rapid progress is being made in developing transgenics in other species.

Three methods are currently in use to create transgenic animals: injection of linear DNA fragments into the pronucleus of one-cell embryos; injection of genetically modified embryonic stem cells into blastocytes; and retroviral infection of pre- or post-implantation embryos.

The transgenics mainly used today in the field of genotoxicity are the bacteriophage lambda-based transgenic mouse models. BigBlue lacI mouse (Stratagene, La Jolla, USA) contains a lambda shuttle vector (bacteriophage) that carries a lacI target and an alpha lacZ reporter gene integrated as a tandem repeat of 40 copies into mouse chromosome 4 (Kohler et al 1991). This model is available in B6C3F1 and C57B1/6 mice. MutaMouse also contains a lambda shuttle vector (bacteriophage) that carries a lacI target and an alpha lacZ reporter gene integrated as a tandem repeat of 40 copies into mouse chromosome 4.

In the field of carcinogenicity the transgenic mouse line TG.AC and the heterozygous p53-deficient (±) transgenic mouse are used. The transgenic mouse line TG.AC (Tennant et al 1996) carries a v-Ha-ras gene fused to the promoter of a foetal (zeta) globin and uniquely responds to chemical carcinogens and tumour promoters by the induction of epidermal papillomas. The heterozygous p53-deficient (±) transgenic mouse (Tennant et al 1996) has only a single wild type p53 allele providing a distinct target for mutagens. This is analogous with man at risk from heritable forms of cancer. The reduction in p53 gene dosage by this germ line first hit increases the probability that a second mutagenic event will cause either loss of p53 tumour-suppressor function or a gain of transforming activity by requiring only a single mutation (in the remaining functional p53 allele). It also creates permissive conditions (i.e. genomic instability) for clonal expansion of cells harbouring mutations in other genes critical to tumourigenesis.

Transgenics are created to address a variety of biological problems, particularly in development and cancer studies. Relatively few such experiments have been reported but they have shown some important implications, and carry the promise of providing much more insight into the complex regulatory circuitry of gene networks.

Of particular interest to mutagenesis and genetic toxicology are transgenic lines carrying shuttle-vector constructs. They are being used to study and quantitate tissue and even cell-specific mutations. They likewise hold great promise for providing a method for improving our capability to recognize with much greater accuracy and certainty chemicals that might pose mutagenic or carcinogenic hazards to man. In addition, analysis by gene knock-out or insertion might advance investigation into underlying mechanisms of carcinogens in-vivo.

Future prospects

The results obtained so far with lacI or lacZ transgenic mice indicate that various organs and tissues, including germinal cells, can be analysed for mutations. Such transgenic mouse models therefore enable direct correlation of mutations—the ultimate molecular endpoint of DNA damage and its attempted repair—with important physiological processes thought to be causally related to mutations, for example cancer and ageing.

An important advantage of these transgenic mice is that studies on the mutagenicity of chemicals can be performed using low doses (Gossen et al 1994). In future multi-endpoint experiments with transgenic mice might be advantageous, for example combining gene mutation in the liver or other organs, micronuclei determination (flow cytometry) in bone marrow or peripheral blood and DNA strand-breaks using comet technology. In these experiments pharmacokinetics and toxicokinetics could also be included.

Future objectives for transgenic systems

The following areas of research interest are not intended to be complete, but are thought to meet the objectives of genetic toxicology. Research efforts might be directed at the creation of new transgenic animals with specific genes whose expression is altered by chemical agents, for which the induced responses are tissue- and organ-specific, and for which a relationship between toxicant exposure in target organs and subsequent development of disease sequelae might be established. For example: new transgenic animals might be developed for the identification and characterization of genes that are involved in modulation of the cellular signalling pathways that lead to the xenobiotically induced health effects; and transgenic animals could be designed to improve our understanding of the correlation between chemical induction or suppression of programmed cell death or apoptosis and subsequent health consequences.

Transgenic animals could be used to study the over-expression or inappropriate expression of xenobiotic inducible genes to enable understanding of the molecular basis of tissue-specific and stage-specific gene expression after exposure to the

chemical agent. New transgenic animals could also be used to study the regulation of DNA repair with emphasis on the identification and characterization of novel systems, and the molecular dissection of the genetic elements responsible for the cellular response. For example, studies might be designed: to investigate cell- and tissue-specific expression of the DNA repair genes induced by xenobiotic agents; and for study of the interactions between environmentally induced repair pathways, transcriptional induction, cell cycle arrest, and DNA replication.

High Throughput Testing

An important milestone in drug development is the decision to administer a candidate drug to man. The available toxicology data are crucial to the decision-making process at this point, both in terms of selecting the best compound and conducting the first clinical studies as safely as possible (Dayan 1991). This has been true for a long time and still is in our age of genomics, proteomics, combinatorial chemistry and high throughput screening. Today the crucial points in the development of a pharmaceutical product occur at a somewhat earlier stage and are called optimum lead identification and candidate selection. The toxicological strategies and technologies contributing to these objectives have to be seen in the context of the need to employ toxicity tests that enable assessment of toxicity endpoints of very low quantities of thousands of compounds in an extremely short time. Therefore, in order to avoid a toxicological and genotoxicological bottle-neck, new, fast and miniaturized genotoxicity test methods have to be developed that can be employed in a higher-throughput manner to predict the toxicity and genotoxicity of drug candidates. This in effect means that genetic toxicology must even more strongly embrace the integration of related disciplines such as molecular biology, biochemistry and biophysics to identify potential surrogate endpoints.

As a very modest start in the direction of micronized genotoxicity testing new bacterial systems have been developed.

Ames microsuspension-test

The aim of this method (Deparade & Arni 1997) is to use the original and widely accepted tester strains but to apply only very small amounts of test substance. The method is based on a simple modification of the *Salmonella* liquid-incubation test (Kado 1983). This modification consists of adding increasing numbers of bacterial cells (approximately 10^9) in a concentrated suspension to liver homogenate mix and test-substance solution, all in a 0.2-mL volume. After incubation for 90 min at 37 °C the mixture is processed according to the standard Ames test protocol. This modification was reported to be 20 times more sensitive than the standard plate-incorporation test and 13 times more sensitive than the normal liquid-incubation protocol.

New Ames test approach

At the beginning of modern genetic toxicology, which was marked by the development of the first *Salmonella typhimurium* tester strains (Ames et al 1975), a new set of genetically altered histidine-requiring *Salmonella* strains paved the way to high throughput genotoxicology.

Meanwhile, Bruce Ames and co-workers (Gee et al 1994) have constructed a set of six new *Salmonella typhimurium* (TA7001 to TA7006) tester strains which identify the six possible base-pair substitution mutations. Each of these carries a unique mis-sense mutation in the histidine biosynthetic operon. In addition to the his mutation, these strains carry different auxiliary features that enhance the mutability of the target his mutation. These include the R factor pKM101, which has the SOS-inducible mucAB system; a deletion of the uvrB component of excision repair; and rfa mutations to increase the accessibility of bulky chemicals to the bacteria. The strains have considerably lower spontaneous reversion frequencies and detect a variety of mutagens at a sensitivity comparable with that of the *Salmonella* tester strains TA100, TA102 and TA104. The low spontaneous frequency of reversion of a mixture of the six tester strains (approximately 10 revertants per plate) also makes possible a simple mutation assay with the mixture of these strains together with TA98 and TA1537, for example in 384-microwell plates. A pH indicator is added to the medium—when the bacteria (revertants) grow, the pH changes and the pH indicator changes from red to yellow. Automated evaluation is possible by spectrophotometry.

Several modern techniques that use very different approaches compared with existing systems can contribute to the further development of high throughput screening for genotoxicity. Some of these techniques are two-dimensional gel electrophoresis, northern and western blot, re-combination techniques, for example PCR, and computer technology. These new technologies offer promising approaches that could lead to reliable, automated, high throughput tools in genotoxicology. The following selection, far from being complete, is intended to give an impression of such approaches.

High throughput testing in mammalian cells

There are several possible means of screening mammalian cells for DNA damage. First are those systems that use normal cell lines and record regulatory changes that are typical for DNA damage. Mammalian DNA-damage-inducible genes are associated with growth arrest and apoptosis (Chiarugi & Ruggiero 1996; Smith & Fornace 1996; Zhan et al 1997). The hypothesis is that the tumour suppressor-gene p53 and the apoptosis suppressor-gene bcl2 modulate (functional antagonism) the effect of dominant oncogenes and that the effect of dominant oncogenes on resistance or sensitivity is dependent on the balance between the expression of p53 and bcl2. In all tissues that have self-renewal capacity there exists a delicate balance between cell production by mitogenesis and cell loss as a result of programmed cell-death or broadly termed apoptosis; this maintains total cell numbers within physiologically appropriate ranges. Apoptosis includes genomic DNA cleavage by endonucleases, chromatin condensation (pyknosis), nuclear fragmentation, proteolysis of cytoskeletal and other proteins, plasma membrane blebbing and cell shrinkage. p53 is activated by genotoxic stress and functions as a cell-cycle check-point by regulating the transcription of at least two sets of genes. One is responsible for transient cell arrest in G1 and the other controls the initiation of apoptosis. Both processes eliminate potential oncogenic mutations, either by proper DNA repair or by inducing damaged cells to commit suicide. bcl2, on the other hand, acts as a functional antagonist of p53 and inhibits apoptosis. c-myc protein interrupts the genetic control of the cell cycle. bcl2 and c-myc inhibit growth-arrest induced by p53. Therefore, the consequences of DNA damage are activation of p53 expression, deactivation of bcl2 expression, induction of growth arrest and apoptosis.

The ML-1 myeloid leukaemia cell system from man can be considered a good example of the use of these principal mechanisms.

ML-1 human myeloid leukaemia cell system

Exposure of ML-1 myeloid leukaemia cells from man to DNA-damaging agents induces dramatic changes in the expression of a variety of gene products (Zhan et al 1997), including increases in: p53 (wild-type); mcl1 (gene of bcl2 family involved in differentiation); growth arrest in G1 (consequence of p53); and DNA damage-inducible genes such as GADD45. There is also a decrease in the proto-oncogene bcl2 (anti-apoptosis gene).

These changes occur as early events in a sequence that culminates in DNA-damage-induced apoptosis. Expression of mcl1, a gene expressed during ML-1 cell differentiation, was found to increase upon exposure of ML-1 cells to various types of DNA-damaging agent. This increase in mcl1 occurred rapidly and was transient, levels of mcl1 mRNA being elevated within 4 h and having returned to near baseline within 24 h.

The 20-year-old example of genetically altered *Salmonella* strains shows that major progress in the field of genetic toxicology is derived from the construction of genetically altered organisms. Therefore, genetically altered mammalian cells should be used to meet the requirements of today's accelerated drug development.

The transcriptional mechanisms by which cells respond to DNA-damaging agents can be used to test for DNA damage (Todd et al 1995; Beard et al 1996). The measurement of gene induction was facilitated by use of different reporter constructs integrated stably into the cell line. These constructs were derived from stress promoters or response elements, or both, isolated from genes associated with DNA-damage responses in cells from man. They were fused to the bacterial reporter gene chloramphenicol acetyl transferase (CAT). The cell-lines generated in this manner contain the promoters or response elements, or both, representing DNA polymerase beta, p53, gadd (growth arrest and DNA damage), c-fos, TPA response element, and tissue-type plasminogen activator.

Two of the so-called CAT-assays are the DNA-damage test in colon carcinoma cell-line (RKO) from man, and the cellular-stress assay in the liver cell-line (HepG2), also from man. These can be used to measure a wide spectrum of stresses, both toxic and non-toxic, such as protein and protein-biosynthesis perturbations and DNA damage. These cellular-stress assays might serve as a means of screening for a variety of substances at the molecular level.

Gene-chip technology

The latest developments in high throughput screening systems for toxicity endpoints using the induction of gene expression are chip systems (affymetrix, synteni) which use DNA-array technology. A DNA array is an array of many single-stranded DNA probes, each bound to a specific location on a solid surface, e.g. silicon. DNA arrays operate on the principle of DNA hybridization. After in-vivo treatment RNA from different organs can be isolated and tested for binding to these DNA probes (genes). Binding and, therefore, the induction of certain genes can be seen by fluorescence tagging. Evaluation is performed by means of computational microscopy. The main problems of such systems are possible artefacts and the scien-

tific interpretation of the results. Reducing the signal-to-noise ratio and determining the variance and sensitivity of these array seem to be the problems that must be overcome before gene-chip results can be taken as a basis for serious decisions.

Computational toxicology

Another completely different approach would be to use only computers instead of performing experiments. This field, denoted computational toxicology, uses computer technology to assess the toxicity of a chemical from its molecular structure. It is based on the axiom that chemical properties can be deduced from information in the chemical structure. Relationships between molecular structure, electron density, metabolic activation and covalent binding of reactive intermediates are important information with regard to the carcinogenic and mutagenic potencies of chemical compounds. Instead of using a biological transformation test to determine the toxicity of a chemical, a computer model is used to predict toxicity data by performing mathematical transformation of a chemical structure. Primarily, there are two fundamental approaches to the prediction of chemical toxicity from molecular structure.

Expert system approach. An expert system is basically a collection of rules derived from the existing subject knowledge and stored in computer memory. There are two kinds: in real expert systems, for example HazardExpert, Oncologic, or Derek, the rules are derived from experts, i.e. reflect man's knowledge and bias; in the second kind, called artificial expert systems, the rules are machine-learned, i.e. the computer system utilizes rules learned from the available data. Artificial expert systems might suffer from the drawback of often finding unreal associations, especially when the rules are formulated from structural patterns poor in information.

QSAR system approach. In principle, a QSAR model is a quantitative relationship between a numerical measure of toxicity and a set of structure descriptors (for example electrophilicity and electrotopology). These structure–activity relationships are generally called quantitative structure–activity relationship (QSAR) models or equations. Searches are performed as complex similarity searches; systems can be either 3-dimensional (e.g. Compact) or 2-dimensional (Topcat).

All computational toxicology systems are based on models developed from closed systems including knowledge or values of activity (toxicity) based on a limited training set of chemicals. The application of these models to all chemicals is simply not prudent and should be restricted to chemicals with a common mode of action. Consequently, it is of utmost importance to ascertain whether the model is applicable to the query chemical presented to it.

Conclusions

When identified mutagens and identified carcinogens are correlated, the result is significantly less than unity. Processes or mechanisms that are not directly genotoxic seem to play a role in carcinogenesis. Whereas short-term genotoxicity test data are still components of the assessment of carcinogenic risk, genetic damage has been recognized as important in its own right, in relation to heritable genetic risk and other health-related effects, such as ageing, reproductive failure and developmental toxicity.

The revolution in molecular biology and genetic analysis over the past 20 years has contributed to the wealth of new information on the complexities of cell regulation, cell differentiation and the carcinogenic process. These technologies have provided new experimental approaches to genetic toxicology assessments, including transgenic cell and animal models. The animal models should tell us more than we have learned from conventional animal models.

The potential exists for the development of more efficient and more relevant genetic toxicology-testing schemes for assessing the safety of man. Delineation of contemporary needs, a modern view of the elements of cancer induction, and an examination of new assays and technologies might provide a framework for integrating new approaches into current schemes for evaluating the potential genetic and carcinogenic risk of chemicals and drugs.

Pharmaceutical and biotechnical companies across the globe are developing novel drugs at an increasing pace. Economic, scientific and social forces are driving them to heighten the speed and efficacy of toxicological assessment of these new pharmaceutical products. Powerful new screening methods have been and are being developed; in conjunction with expanded compound libraries these can and will expand the knowledge-base of all parties in the dynamic and expanding field of molecular-level toxicology and genotoxicology.

It is certain that in the near future new technologies will alter toxicology and genotoxicology far more than in the last 10 years. It can also be assumed that by using the new methods described above genotoxicology as a science of its own will lose its accustomed and slightly exotic place at the

edge of toxicology, biochemistry and molecular biology.

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References

Adler, I., Wyrobek, A. (1997) Aneuploidy and chromosomal aberrations detected in sperm of rodent and men using multicolour FISH technology. ICEM Satellite Meeting: Cancer and Genetic Risk Assessment: Low Dose-Effect Studies, September 1997, Heidelberg, Germany

Ames, B. N., McCann, J., Yamasaki, E. (1975) Methods for detecting carcinogens and mutagens with the *Salmonella/* mammalian-microsome mutagenicity test. Mutat. Res. 31: 347–364

Beard, S. E., Capaldi, S. R., Gee, P. (1996) Stress responses to DNA damaging agents in the human colon carcinoma cell-line, RKO. Mutat. Res. 371: 1–13

Boveri, T. H. (1929) The Origin of Malignant Tumours, Williams & Wilkins, Baltimore, MD

Chiarugi, V., Ruggiero, M. (1996) Role of three cancer 'master genes' p53, bcl2 and c-myc on the apoptotic process. Tumori 82: 205–209

Dayan, A. D. (1991) The limitations of animal studies: what can and cannot be predicted for man. Drug Inf. J. 25: 165–170

Deparade, E., Arni, P. (1997) Ames Microsuspensions-Test ein mikrobieller Mutagenitätstest zur Prüfung geringer Substanzmengen. Poster GUM Jahrestagung 1997, Basle, Switzerland

Elespuru, R. K. (1996) Future approaches to genetic toxicology risk assessment. Mutat. Res. 365: 191–204

Gee, P., Maron, D. M., Ames, B. N. (1994) Detection and classification of mutagens: a set of base-specific *Salmonella* tester strains. Proc. Natl Acad. Sci. USA 91: 11606–11610

Gossen, J. A., de Leeuw, W. J. F., Vijg, J. (1994) LacZ transgenic mouse models: their application in genetic toxicology. Mutat. Res. 307: 451–459

ICH (1997) Harmonized Tripartite Guideline, Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals, Recommended for Adoption at Step 4 of the ICH Process on 16 July 1997 by the ICH Steering Committee (Final Draft)

Kado, N. Y., Langley, D., Eisenstadt, E. (1983) A simple modification of the *Salmonella* liquid-incubation assay. Increased sensitivity mutagens in human urine. Mutat. Res. 121: 25–32

Kirsch-Volders, M., Elhajouji, A., Cundari, E., Van Hummelen, P. (1997) The 'in vitro' micronucleus test: a multi-endpoint assay to detect simultaneously mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction. Mutat. Res. 392: 19–30

Kohler, S. W., Provost, G. S., Fieck, A., Kretz, P. L., Bullock, W. O., Sorge, J. A., Putman, D. L., Short, J. M. (1991) Spectra of spontaneous and mutagen-induced mutations in the lacI gene in transgenic mice. Proc. Natl Acad. Sci. USA 88: 7958–7962

MacGregor, J. (1997) ICEM 1997, Toulouse, France, September 1997

Parry, J. M. (1997) Mutation analysis using the restriction site mutation assay. ICEM Satellite Meeting: Cancer and Genetic Risk Assessment: Low Dose-Effect Studies, September 1997, Heidelberg, Germany

Preston, R. J., Au, W., Bender, M. A., Brewen, J. G., Carrano, A. V., Heddle, J. A., McFee, A. F., Wolff, S., Wassom, J. S. (1981) Mammalian 'in vivo' and 'in vitro' cytogenetic assays: a report of the USEPA's Gene-Tox Program. Mutat. Res. 87: 143–188

Russel, W. L. (1951) X-ray-induced mutations in mice, Cold Spring Harbor Symp. Quant. Biol. 16: 327–356

Salamone, M., Heddle, J., Stuart, E., Katz, M. (1980) Towards an improved micronucleus test. Studies on 3 model agents, mitomycin c, cyclophosphamide and dimethylbenzanthracene. Mutat. Res. 74: 347–356

Smith, M. L., Fornace, A. J. (1996) Mammalian DNA damage-inducible genes associated with growth arrest and apoptosis. Mutat. Res. 340: 109–124

Storer, R. D., Kraynak, A. R., McKelvey, T. W., Elia, M. C., Goodrow, T. L., DeLuca, J. G. (1997) The mouse lymphoma L5178Y Tk ± cell-line is heterozygous for a codon 170 mutation in the p53 tumor suppressor gene. Mutat. Res. 373: 157–165

Tennant, R. W., Spalding, J., French, J. E. (1996) Evaluation of transgenic mouse bioassays for identifying carcinogens and noncarcinogens. Mutat. Res. 365: 119–127

Tice, R. (1997) Detection of DNA breakage in sperm by application of the comet assay. ICEM Satellite Meeting: Cancer and Genetic Risk Assessment: Low Dose-Effect Studies, September 1997, Heidelberg, Germany

Todd, M. D., Lee, M. J., Williams, J. L., Nalezny, J. M., Gee, P., Benjamin, M. B., Farr, S. B. (1995) The CAT-Tox (L) assay: a sensitive and specific measure of stress-induced transcription in transformed human liver cells. Fundam. Appl. Toxicol. 28: 118–128

Vainio, H. (1995) Molecular approaches in toxicology: change in perspective. J. Occup. Environ. Med. 37: 14–18

Watanabe, P. G. (1997) Changing times require changing behaviour. Fundam. Appl. Toxicol. 37: 101

Zhan, Q., Bieszczad, C. K., Bae, I., Fornace, A. J., Craig, R. W. (1997) Induction of BCL2 family member MCL1 as an early response to DNA damage. Oncogene. 14: 1031–1039