K.S. Rao, D.V.M., Ph.D.; Yong Xu, M.D., M.Ph.; Ellen Shaw, M.S.; and **Joseph W. Parton**
MicaGenix, Inc., 2000 West Main Street, Greenfield, IN 46140 USA

Mutagenicity Testing Applied for Regulation of Developing Products

Background

Newly discovered products (pharmaceuticals, foods and food additives, and other chemicals) need a thorough investigation of their safety and efficacy to human health before release into the market. Most regulatory agencies, including the U.S. Food and Drug Administration (FDA) require a series of toxicological tests, including mutagenesis testing.

Mutagenesis refers to those changes in the genetic material in cells brought about spontaneously either by chemical or by physical means whereby successive generations differ in a permanent and heritable way from their predecessors. Current scientific knowledge overwhelmingly supports the concept that many chemicals possess mutagenic properties that present a potential genetic hazard to future generations, as well as a potential cancer risk to the present one. Chemicals that exert adverse effects through interaction with the genetic material, deoxyribonucleic acid (DNA), are called genotoxic (1).

Genetic toxicology is concerned primarily with the mutation effects of chemicals. Mutation refers to a genetic alteration in somatic (body) or germ cells. The mutations in somatic cells may contribute to various defects including cancer, while the mutations in germ cells cause potential genetic disease in future generations (2,3). While the relationship between exposure to particular chemicals and carcinogenesis is established for man, a similar relationship for heritable defects has been difficult to prove. Genotoxicity tests have been used mainly for prediction of carcinogenicity (FDA Guidance for Industry, 1997).

The classical toxicological procedures do not lend themselves to meaningful ways for assessing genetic toxicity of potential New Chemical Entities. The need for mutagenicity tests that are quicker, lower cost and highly sensitive thus became urgent. Over the past two decades, a wide variety of systems have been investigated and found to offer good means of assessing the mutagenic potential of new compounds in a cost effective way. Increased understanding of the nature and function of genetic material and its response to disturbance have made this development possible. It is now clear that DNA is the basic carrier of genetic information common to all living cells and that damage to DNA is the fundamental mechanism of induced mutation (4).

Government agencies (FDA and others) are confronted with regulation of new pharmaceutical and biotech products, with risk estimation of putative genotoxic or carcinogenic compounds. The strategy for assessment of human health risks of xenobiotic agents requires genotoxicity testing. Here we discuss strategies and tests for mutagenic assessment that are the primary focus of MicaGenix at this time.

The MicaGenix laboratory for Mutagenicity Testing was founded in 2002 by Joseph W. Parton, after 35 years of experience at Eli Lilly and Company. From the very beginning, the goal of the new laboratory was to provide testing and advice in the field of mutagenicity assessment and to offer an expert service to its clients.

Definitions

1. In the present context, commonly found definitions of the terms *mutagenic*, *mutagen*, *mutations* and *genotoxic* are used. *Mutation* is defined here as a permanent change in the amount or structure of the genetic material in a cell.

2. The term *mutation* applies both to heritable genetic changes that may be manifested at the phenotypic level, and to the underlying DNA modifications when known (including, for example, specific base pair changes or chromosomal translocations). The terms *mutagenic* and *mutagen* will be used for agents giving rise to an increased occurrence of mutations in populations of cells and/or organisms.

3. The more general terms *genotoxic* and *genotoxicity* apply to agents or processes that alter the structure, information content, or segregation of DNA, including those that cause DNA damage by interfering with normal replication processes, or which in a non-physiological manner (temporarily) alter its replication. Genotoxicity test results are usually taken as indicators for mutagenic effects.

What is Mutagenicity Testing?

Chemical or physical impacts can result in a fixed alteration in the genetic material (mutation) in cells, which lead to lethal or heritable defects.

In order to identify those substances before they can cause harm, genetic tests and screening procedures have been developed (mutagenicity tests). The scientific field of mutagenicity testing develops and uses these tests to screen newly synthesized chemicals for those that induce mutations. This process is called mutagenicity testing.

Using a variety of genetic endpoints both *in vitro* and *in vivo*, the genotoxic potential of a chemical can be assessed. Two basic categories of endpoint, gene mutation and chromosomal alterations are believed to be responsible for induction of somatic (including carcinogenic) as well as heritable defects. Induction of damage by chemicals can be specific or preferential for one or the other endpoints. Accordingly, it is considered necessary to develop a testing strategy that includes tests for both gene

mutation and chromosomal aberration (5). The list of mutagenicity test procedures for which short descriptions are given consists mainly of well-validated examples that are most often used and requested. Other assays as alternative tools for a specific purpose can also be offered.

Mutagenicity Testing Strategy

Present regulatory requirements around the world vary widely both in the number of tests required and the weight placed upon them. A need for a unified approach is clearly recognized. Through the International Conference of Harmonization process (6), a uniform testing scheme was developed for the use of genetic toxicology in toxicological evaluation. To this end, government agencies recognize genotoxic activity as a potential hazard for adverse human health effects, and accordingly, genotoxicity is a *bona fide* toxicological endpoint. It is likely that a chemical that results in a mutagenic response in testing will possess the potential to manifest this mutagenic activity as some adverse health effect in humans. There are three questions that must be answered in the evaluation of mutagenic activity.

1. Is the compound mutagenic in some biological system? This can be answered by determining its mutagenic potential in the sensitive *in vitro* short-term tests.
2. Is the compound a mammalian mutagen? This can be answered through prudent use of *in vivo* tests for mutagenic activity in mammalian somatic cells.
3. Is the mutagenic activity of the compound expressed as any adverse health effects? To determine this, it is necessary to determine if cancers are induced in the appropriate bioassays for carcinogenicity.

Selection of Test Systems

It is now widely accepted that no single test selected from the wide range available can be expected to fulfill the requirements of simplicity, rapidity and low cost and yet be absolutely accurate in predicting genotoxic effects to humans. However, there is considerable and growing evidence that a judicious combination of test procedures affecting different genetic endpoints will detect the majority of potential mutagens (7).

A set of *in vitro* and *in vivo* genotoxicity tests with different end-points have been established for assessment of genotoxic potential of chemicals as shown in ***T1***.

In this review, we restrict our discussion to the tests considered valid and necessary by the ICH process (6), which in turn is accepted by all government agencies worldwide.

Proposed Mutagenicity Tests

It is generally acknowledged that the above requirements and considerations are not met by any single test, but only by a well-selected combination of procedures. However, the combination of tests applied should in each case depend on the specific characteristics of the substance to be tested. Based on current knowledge, a system using four categories of tests is proposed as an appropriate approach to determine the mutagenic potential of a chemical. Normally one test from each of the following four categories should be selected. Tests from at least three of the four categories must be completed for a full assessment of mutagenicity.

a) Test for Gene Mutations in Bacteria - Ames Test

This is the most widely used test for assessing the mutagenic properties of chemicals. The Salmonella typhimurium histidine (his) reversion system is a microbial assay that uses a set of histidine-requiring strains of bacteria to detect frameshifts and base pair substitution mutations (8). Treatment with mutagens can induce the mutations in the histidine operon and shift growth of the strains from a histidine-requiring to a histidine-independent pattern. The change in the growth phenotype represents an indicator of mutagenic response. The role of metabolic activation on the mutagenic effect of chemicals can be addressed by using metabolic activation fraction of rat liver homogenate mimicking *in vivo* situation.

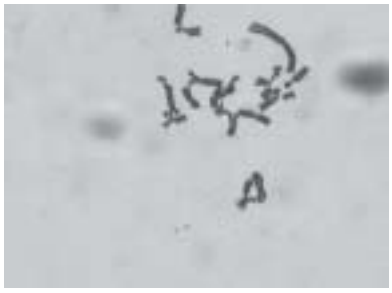
b) Test for Chromosomal Aberrations in Mammalian Cells

In Vitro

The chromosome aberration assay in cultured cells has been widely used for many years, and it has proved to be a useful and sensitive test for detection of genotoxic agents. The damage is scored by microscopic examination of chromosomes in mitotic metaphase cells (***F1***). Tests are carried out with and without extrinsic metabolic activation (9).

The cultured Chinese Hamster Ovary (CHO) cells or human

F1. Chromosome aberrations in Chinese hamster ovary cells.



T1. Tests and End-Points

Mutagenic Process	End-Points	Testing
Pre-mutagenic lesions	Interaction of chemical and DNA	DNA adducts
DNA damage	DNA damage and repair	Comet assay
Fixed in gene mutation	Gene mutation including base pair substitutions and frameshifts	Ames test *E. coli* WP2 tryptophan reversion assay *In vivo* genetic assays
Alteration of DNA	Chromosome aberrations, DNA breakage	Chromosome aberration assay Micronucleus assay

lymphocytes are treated with chemicals in the presence or absence of metabolic activation. Depending on the phase of the cell cycle, chromosome mutations will manifest as chromosome-type aberrations (when they occur during the G1- or S-phase), or as chromatid-type aberrations when the mutations occur during the G2-phase. Chromosome-type aberrations involve both sister chromatids at identical loci. Chromatid-type aberrations are changes in single chromatids or breakage and reunion involving chromatids of different chromosomes. Numerical aberrations are variations of the chromosome number of the cell leading to aneuploidy or polyploidy.

c) In Vivo *Genetic Assays*

The relevance of this test is that a positive result found in bacteria can be additionally studied in a system that has the complex eukaryotic chromosomal structure. This structural complexity also allows the possibility of detection of mutations arising through mechanisms that cannot occur in the simple bacterial genome. Suitable tests include those using mammalian cells designed to detect induction of mutations at specific loci such as those coding for the enzymes hypoxanthine-guanine-phosphoribosyl -transferase or thymidine kinase (10).

d) In Vitro *and* In Vivo *Micronucleus Assay*

The micronucleus test is used for detection of damage to the chromosomes or the mitotic apparatus induced by chemicals (11). Micronuclei are small particles consisting of acentric fragments, or entire chromosomes that lag behind at anaphase of cell division (***F2***). After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm. The assay has been developed into *in vitro* and *in vivo* processes to detect clastogens and aneugens.

In vivo micronucleus assay is a mutagenic assay required by regulatory agencies, and involves microscopic examination of cytological preparations of polychromatic erythrocytes obtained from the bone marrow of animals. The *in vivo* assay takes into account whole animal processes, like absorption, tissue distribution, metabolism and excretion of a foreign chemical and its metabolites, and repair of lesions. Moreover, in a regulatory context, a relevant negative *in vivo* result from an adequately performed and relevant test can essentially negate a positive *in vitro* mutagenic test, at least in terms of its impact to potential health concerns under the use conditions of the drug in question.

An *in vitro* micronucleus assay using cultured cells has been developed (12). This assay is more easily scored than the chromosome aberration assay and utilizes relatively small amounts of test article, thus requiring less time to make an assessment of mutagenic potential of a chemical. Therefore, this assay has been widely used as an alternative means to screen for mutagens. CHO cell cultures are exposed to the test chemical in the presence and absence of metabolic activation system. The treated cells are allowed to form binucleated cells due to exposure to cytochalasin B (***F3***). The micronuclei in the binucleated cells are scored using light microscopy as the indicator of mutagenic response.

e) Comet Assay: An Alternative Tool to Assess DNA Damage and DNA Repair

The single cell gel electrophoresis (Comet assay) can be used to investigate the genotoxicity of industrial chemicals, biocides, agrochemicals and pharmaceuticals. Comet assay detects DNA strand breaks which, when subjected to electrophoresis, will result in migration of DNA fragments out of the nucleus to form the tail of a comet-like structure (***F4***). The extent of migration of DNA fragments is an indication of DNA damage that can be quantified. The comet assay is amenable for both *in vitro* (in any cell type) and *in vivo* or ex-*in vivo* in any species and in any target tissue. As a result, the comet assay can be incorporated in any routine toxicology experiments, which can add value without adding any extra animals. During early drug development, robust genotoxicity screening assays are required that reliably predict the outcome of the time- and resource-consuming regulatory tests. In this respect, the Comet assay is a promising tool because it is rapid, simple to perform, and requires only a small amount of test substance (13).

The *in vivo* alkaline comet assay can be applied to any tissue in the given *in vivo* model and provide a result based on single cells. Therefore, the assay has potential advantage over other *in vivo* genotoxicity test methods that are reliably applicable to rapidly proliferating cells only (bone marrow cells) and/or have been validated preferentially in a single tissue only (the liver unscheduled DNA synthesis assay). Using the comet assay, 208 chemicals selected from the IARC monographs and US NTP carcinogenicity database were investigated (14). The *in vivo* comet assay detected a positive response in 110 of 117 rodent genotoxic carcinogens and a negative response in 6 of 30 rodent non-genotoxic carcinogens. Also, 32 of 54 rodent carcinogens that did not induce micronuclei in bone marrow were found positive in the *in vivo* comet assay. Therefore, *in vivo* comet assay can be an alternative and powerful tool to detect genotoxic chemicals for regulatory purposes. Furthermore, this assay has been used in human clinical trials for detecting genotoxic or protective effects of chemicals.

The *in vitro* comet assay using cultured cells has been used for testing of genotoxic chemicals and for mechanistic genotoxicity testing. The high sensitivity of the comet assay compared to the chromosomal aberration and micronucleus tests, and the need for only very small amounts of test chemical, makes this assay an alternative tool to screen and verify the genotoxic property of chemicals. Using modified protocols, the *in vitro* comet assay can also be applied for DNA repair and apoptosis. If appropriate, extrinsic metabolic activation may be incorporated into this test.

Evaluation of Mutagenic Activity

In evaluating chemicals for mutagenic activity, a number of factors will be considered:

1. genetic endpoints (e.g., gene mutations, structural or numerical chromosomal aberrations) detected by the test systems;

2. sensitivity and predictive value of the test systems for various classes of chemical compounds;

3. number of different test systems used for detecting each genetic endpoint;

4. consistency of the results obtained in different test systems and different species;

5. aspects of the dose-response relationship; and

6. whether the tests are conducted in accordance with appropriate test protocols agreed upon by experts in the field.

Most compounds tend to induce either point mutations or chromosome aberrations or both, although there are no drugs that induce exclusively either gene mutations or chromosome aberrations. This implies that one has to test New Chemical Entities for both endpoints.

Interpretation of the Results

The objective of the mutagenicity testing procedures is to establish with reasonable certainty whether a substance possesses mutagenic properties or not. Following from this is a second and quite separate issue, which is the significance of the obtained results in terms of genetic hazard to man. If all results indicate convincingly that a substance has no effect in any of the tests, then it would seem reasonable to conclude that the possibility of mutagenic hazard is of an acceptable low order (although it may be considered evidence of absence of carcinogenic potential). If all results both *in vitro* and *in vivo* indicate that the compound has mutagenic properties, this would argue strongly for the existence of a risk to humans. Often the results of these tests are not uniform. This is to be expected, since the tests are designed to have different end points and/or different characteristics for metabolic activation. In such cases, the significance of positive and negative results is to be judged not by their number but by their nature. For instance, for the tests outlined, a positive result in an *in vivo* test deserves more weight than a positive bacterial test. This difference does not apply to negative results, implying that one negative *in vivo* test does not necessarily invalidate a series of positive results obtained by *in vitro* testing.

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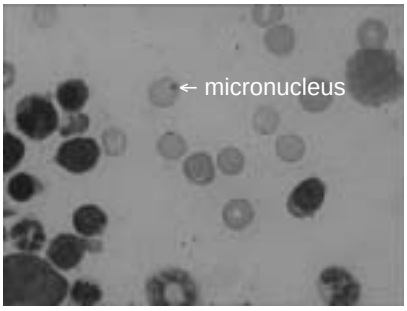
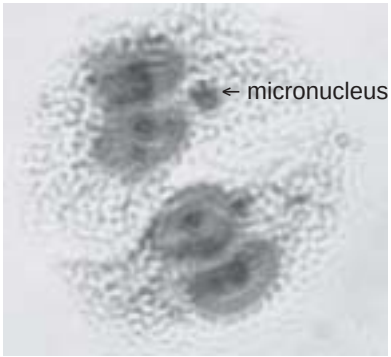
F2. Bone marrow micronuclei.



F3. *In Vitro* micronucleus in Chinese hamster ovary cells - a binucleated cell.



F4. Comet assay assembly.

Extended Dynamic Range Acquisition
Avoids saturation of the head and wash-out of the tail. Necessary for *quantitative* analysis of comet images.

Adaptive Background Correction
Based on each comet image, needed for accurate comparison of comet images.

Objective Delineation of Comet Heads and Tails
Handles cases of necrosis and apoptosis (mostly tails). User intervention not required.

Quantitative Measures
Tail length, tail area, tail moment, tail moment arm, tail moment of inertia, integrated cell intensity, percent DNA in the tail, fragmentation.

GLP Compliance Support
Security levels, password access, audit trail logs.