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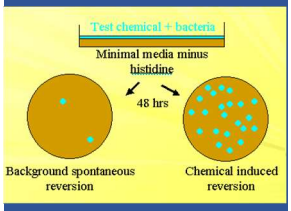

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"It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change."

– Charles Darwin

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Sapna Gupta

toxgurukul.india@gmail.com

Editor.Toxgurul@gmail.com

GENOTOXICITY ASSESSMENT OF DEVELOPMENTAL PRODUCTS

K.S. Rao[1], M.V.Sc., Ph.D., DABT and Shahnaz Akhtar[2], Ph.D., DABT

[1]Eurofins Advinus, Bangalore;

[2]Sun Pharma Advanced Research Company Limited, Vadodara

Historical Perspective

Modern genetic toxicology studies were started by H.J. Muller in 1927, his work showed that X-rays could induce mutations in the fruit fly, Drosophila. In 1946, Charlotte Auerbach and colleagues reported chemical (mustard gas) induced mutations in Drosophila were phenotypically similar to the mutations induced by X-rays in Muller's experiments. Extrapolation of similar effects to human were not clear until William Russell (1951) reported that X-rays also induce mutations in mouse-specific-locus mutation assay. Late in 1950's, the Nobel Prize winners Hermann Muller and Joshua Lederberg commented on the potential public health hazards of mutagenic effects of chemicals and suggested the development of a regulatory mutagenicity testing program.

Development of gene mutation assay using *Salmonella typhimurium* by Bruce Ames *et al* in 1975 was the most considerable progression in the field of genetic toxicology. In next two decades, research in Genetic Toxicology evolved remarkably. Genetic toxicologists investigated the induction of mutations and chromosomal alterations in somatic and germ cells, following exposures to physical and chemical mutagens, using prokaryotic and mammalian cell lines, primary cultures and *in-vivo* test systems. The importance of mutations and chromosomal alterations for human health is evident from their roles in genetic disorders, including birth defects and cancer. In last decade, genetic toxicology has moved from just assessment of genotoxic potential to more mechanistic approaches.

A. Definitions and Basic Concepts Related to Genetic Toxicology

Genotoxicity: A broad term that refers to any deleterious changes in the genetic material regardless of the mechanism by which it is being induced. It delineates all types of DNA or chromosomal damage, including DNA breaks, adducts, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable (transmissible) chromosomal damage.

Aneugen: Any substance or process which interacts with the components of the mitotic and meiotic cell division cycle and leads to aneuploidy in cells or organisms.

Aneuploidy: A numerical deviation from the modal number of chromosomes in a cell or organism.

Clastogen: An agent that produces structural breakage of chromosomes, usually detectable by light microscopy.

Mutagenicity: A detectable permanent change within a single gene or its regulating sequences. The changes can be point mutations, insertions, or deletions. 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.

Micronuclei: Small fragments of entire nuclear chromosome, separate from and in addition to the main nuclei of cells, produced during telophase of mitosis (meiosis).

S9 liver fractions: Supernatant of liver homogenate after 9000g centrifugation, i.e., rat liver extract.

S9 mix: Mix of the liver S9 fraction and cofactors necessary for cytochrome p450 metabolic enzyme activity.

Metabolic Activation: Not all the test substances interact directly with DNA to exert the genotoxic or mutagenic effects. Mostly these chemicals need to be transformed into a reactive metabolite to cause a genotoxic effect. Most commonly used test systems used in these assays lack metabolic enzymes, and are not able to convert the test substance to reactive metabolite. Therefore, metabolic activating system (S9 fraction) is prepared by homogenized livers of rats pretreated with agents, which induce the P450 mixed function oxidase ("phase I") system. In early 1970s Marvin Legator at the FDA used this concept to develop a mouse host-mediated assay. In this procedure, they injected Ames's *Salmonella* strains into the peritoneal cavity of a mouse, which was then treated by a different route with a non-mutagenic chemical that could be metabolized to a mutagen. The *in-vivo* activated mutagen came into contact with the *Salmonella* cells in the peritoneal cavity and mutagenicity was determined by removing the bacteria, plating them, and determining the mutation frequency. Although relatively insensitive, this procedure demonstrated the importance of mammalian metabolic activation for the mutagenicity of a number of carcinogens. In the absence of practical alternatives, rat liver S9 remains the most common approach, particularly when screening compounds for which there are no preliminary data on which to base an alternative approach.

Concurrent Positive Control: Concurrent positive controls (known genotoxic substances) should be used in all *in-vitro* and *in-vivo* genotoxicity assay to demonstrate the sensitivity of the assay on the day it is performed. Concentration/dose of the positive control used should result in a reliable, reproducible and detectable increase over background. In order to demonstrate the ability of the test system to efficiently detect DNA damage, gene mutations and/or chromosomal aberrations depending on the test, and in the case of *in-vitro* tests, the effectiveness of the exogenous metabolic activation system. Therefore, positive control responses should be observed at concentrations or doses that produce weak or moderate effects that will be detected when the test system is optimized, but not so intense that positive responses will be seen in sub-optimal test systems, and immediately reveal the identity of the coded samples for blind scoring to the scorer.

Once, the laboratory has established competence in said genotoxicity test, for *in-vitro* assays use of positive control can be restricted to metabolic activation only (when it is done concurrently with the non-activated test) and for *in-vivo* studies, positive control can be tested periodically (e.g. annually, or less depending upon the requirements).

Consideration of Cytotoxicity for Selection of Doses/Concentrations of Genotoxicity Assays: For selecting the appropriate concentrations/doses for genotoxicity assays one of the important criteria is the cytotoxicity potential of the test substance. As cytotoxicity can involve mechanisms other than direct DNA damage to produce 'false positive' results. Hence, highest dose selected for *in-vitro* and *in-vivo* assays should not be severely cytotoxic as per the criteria defined for each assay.

Historical Control Data: Historical control data for each assay and for each laboratory plays an important role for interpretation of results of genotoxicity assays. Historical control data (both negative and positive) should be compiled separately for each genetic toxicology test type, species, strain, tissue, cell type, metabolic condition, treatment and sampling time, route of exposure individually, as well as for each solvent or vehicle within each laboratory.

B. Early Regulatory Developments

Following the publication series of Dr. Ames's lab in the 1970s, there was an explosion of interest among research organizations, industries, and, most significantly, government regulatory agencies to identify carcinogens using mutagenicity tests. Soon afterwards, the Environmental Protection Agency (EPA) promulgated a tier-testing approach to genetic toxicity

assays in support of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA), listing an extensive roster of *in-vitro* and *in-vivo* tests covering a range of genetic endpoints. The tier-testing approach was recommended as the most cost-effective and efficient procedure for screening many chemicals and minimizing large-scale animal testing. The more expensive and time-consuming tests would be performed only on chemicals that were known to be mutagens from the initial lower-tier *in-vitro* screening tests.

C. Organized Product Development

Genetic toxicity testing is a necessary and pivotal component of product development and registration. 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 genotoxicity testing.

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, cheaper and more sensitive thus became a dire need over a period. Over the past two decades, a wide variety of systems have been investigated and found to offer good means of assessing the genotoxic potential of new compounds in a cost-effective manner. Increased understanding of the nature and function of genetic material and its response to disturbance has 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.

Government agencies (FDA and others) are confronted with regulation of new pharmaceutical agents with risk estimation of putative genotoxic or carcinogenic compounds. The strategy for assessment of human health risks of xenobiotic substance requires genotoxicity testing. Here we will discuss strategies and tests for mutagenic assessment which are critical in product development.

D. Genotoxicity Assessment

In order to identify the genotoxic substances before they can harm, a set of *in-vitro* and *in-vivo* genotoxicity tests with different endpoints have been established as shown in Table 1 below:

Table 1: 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	a) Comet assay b) Unscheduled DNA Synthesis
Fixed in gene mutation	Gene mutation including base pair substitutions and frame shifts	a) *In-vitro* Bacterial Reverse Mutation Test b) *In-vitro* Mammalian Forward Mutation Test c) *In-vivo* transgenic mutation assays
Alteration of DNA	Clastogenicity, Aneuploidy	a) Chromosome aberration assay b) Micronucleus assay c) Mouse Lymphoma Assay d) Sister Chromatid Exchange

Accordingly, number of testing guidelines were developed for genotoxicity assessment. Table 2, enlists the various currently effective guidelines recommended by OECD for genotoxicity assessment has been compiled:

Table 2: OECD Guidelines for Genotoxicity Assessment

Study type	Test Name	OECD
In-vitro	Bacterial reverse mutation test (Ames test)	471
	In-vitro mammalian chromosome aberration test	473
	In-vitro mammalian cell micronucleus test	487
	In-vitro mammalian cell gene mutation test using Hprt and Xprt locus	476
	In-vitro mammalian cell gene mutation test using Thymidine Kinase gene	490
In-vivo	Mammalian erythrocyte micronucleus test	474
	Mammalian bone marrow chromosome aberration test	475
	Rodent Dominant lethal test	478
	Mammalian spermatogonial chromosome aberration test	483
	Mouse heritable translocation assay	485
	Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in-vivo*	486
	Transgenic rodent somatic and germ cell gene mutation assay	488
	In-vivo mammalian alkaline comet assay	489

E. Testing Strategy for Genotoxicity Studies

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 aberrations are believed to be responsible for induction of somatic (including carcinogenic) as well as heritable defects. Induction of damage by chemicals can be specific to one or the other endpoints. 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 accurate in predicting genotoxic effects to humans. However, there is considerable and widely accepted evidence that a judicious combination of test procedures affecting different genetic endpoints will help identifying potential genotoxicants. Therefore, it is considered necessary to develop a testing strategy that includes tests for both gene mutation and chromosomal aberration.

Global harmonization process was first started in 1991 in Brussels, Germany. It is a tripartite agreement between European Community (EC), USA and Japan. The main objective of International Conference on Harmonization (ICH) is to overcome the regional disparities and harmonize international standards for pre-clinical safety studies of testing pharmaceuticals products. In 1992, genotoxicity gained the ICH interest and the established genotoxicity working group identified more than 60 strategic and technical issues, which were substantially different between the regulatory authorities of the USA, the European Union and Japan. Currently, a participatory approach has been devised by the ICH consisting of representatives from pharmaceutical industry, scientists, academia and regulatory authorities to develop unified guidelines for toxicological testing. A tiered approach is applied in regulatory genotoxicity testing. In the first step, highly sensitive *in-vitro* assays are used to identify test compounds that have high intrinsic genotoxic activity. In the second step, specific *in-vivo* tests are performed to check the reliability of the *in-vitro* results for the *in-vivo* conditions. These *in-vivo* mutagenicity studies are also included because some genotoxicants can only be detected *in-vivo* after metabolic activation.

International Conference of Harmonization process (ICH) recommends a standard battery of tests for pharmaceuticals to detect their genotoxicity. The ICH guidance optimizes the standard battery test for genetic toxicology and provides guidelines on the interpretation of results. These guidelines help improve risk characterization for carcinogenic effects which in turn is accepted by all government agencies worldwide.

The following two options for the standard battery are considered equally suitable:

Option 1

i. A test for gene mutation in bacteria.
ii. A cytogenetic test for chromosomal damage (the *in-vitro* metaphase chromosome aberration test or *in-vitro* micronucleus test), or an *in-vitro* mouse lymphoma Tk gene mutation assay.
iii. An *in-vivo* genotoxicity test, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase arrest during mitosis.

Option 2

i. A test for gene mutation in bacteria.
ii. An *in-vivo* assessment of genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second *in-vivo* assay. Typically, this would be a DNA strand breakage assay in liver, unless otherwise justified.

F. Suggested Mutagenicity Tests

The list of genotoxicity test procedures for which short descriptions are given in this section consists mainly of well validated examples that are most often used and requested. Other assays as alternative tools for specific purposes may also be performed. In this review, we restrict our discussion to the tests considered valid and necessary by the ICH process.

a) Test for Gene Mutations in Bacteria -Ames Test

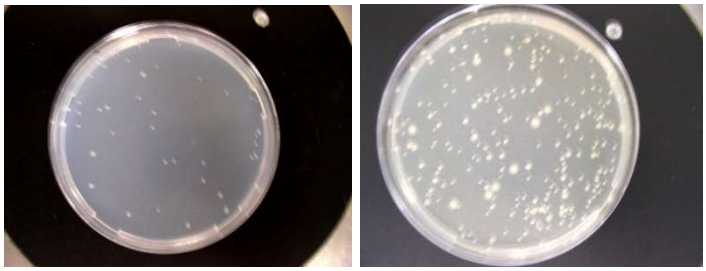
The Bacterial reverse mutation test was developed by Bruce Ames thus it is named as Ames test. This is the most widely used test for assessing the mutagenic properties of chemicals. The amino acid requiring strains of *Salmonella typhimurium* and *Escherichia coli* are used to detect the mutation. The recommended set of bacterial strains includes *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 or TA97 or TA97a; and either *Salmonella typhimurium* TA102 or *Escherichia coli WP2 uvrA* or *Escherichia coli WP2 uvrA* (pKM101). These strains can detect base substitution and frame shift mutations. Each strain contains identified mutations in a gene at the reporter locus for biosynthesis of amino acid (i.e., histidine [His] or tryptophan [Trp], for *Salmonella typhimurium* and *E. coli* respectively). These mutations prevent bacterial growth in the absence of the amino acid in the growth medium. Exposure to mutagens may induce a second mutation (a reversal) that will restore the wild type DNA sequence and the functional capability of the bacteria to synthesize the essential amino acid, and thus, to grow on medium without the required amino acid. The change in the growth phenotype

represents an indicator of mutagenic response. Cells in which this second, function restoring mutation (reversal) has occurred are called revertant and for the test method, bacterial colonies are counted. Consequently, the Ames test is termed a "reverse mutation assay".

The maximum dose level recommended is 5000 µg/plate (or 5 µL/plate for liquid test substance) when not limited by solubility or cytotoxicity. If dose related cytotoxicity or mutagenicity is noted, irrespective of solubility, the top dose scored should be based on cytotoxicity detected by a reduction in the number of revertant colonies, and/or clearing or diminution of the background lawn.

A single bacterial mutation test is considered sufficient when it is clearly negative or positive, and carried out with a fully adequate protocol including all strains with and without metabolic activation, a suitable dose range that fulfills criteria for top dose selection, and appropriate positive and negative controls. Equivocal or weak positive results might indicate that it would be appropriate to repeat the test, possibly with a modified protocol such as appropriate spacing of dose levels.

Fig 1: Colony Growth in Ames test



A: Control **B: Mutagenic Compound**

b) *In-vitro* mouse lymphoma TK gene mutation assay

This assay identifies substances that cause gene mutations at the thymidine kinase (TK) reporter locus. It uses L5178Y $TK^{+/-}$ 3.7.2C cell line. Genetic events detected using the TK locus include both gene mutations (point mutations, frame shift mutations, small deletions) and chromosomal events (large deletions, chromosomal rearrangements and mitotic recombination).

The autosomal and heterozygous nature of the TK gene in the cell line enables the detection of cells deficient in the enzyme TK following mutation from $TK^{+/-}$ to $TK^{-/-}$. Hence, it is a forward mutation assay. Treating cells with the test substance, followed by an incubation period that provides sufficient time (termed the expression time) for the newly induced mutants to lose their functional TK enzyme. The cell population is cloned in the presence and absence of the selective agent Triflurothymidine (TFT) for the enumeration of mutant cells and the measurement of cloning efficiency, respectively, in order to calculate a mutant frequency. Cells deficient in TK enzyme due to mutation $TK^{+/-}$ to $TK^{-/-}$ are resistant to the cytotoxic effects of pyrimidine analogue

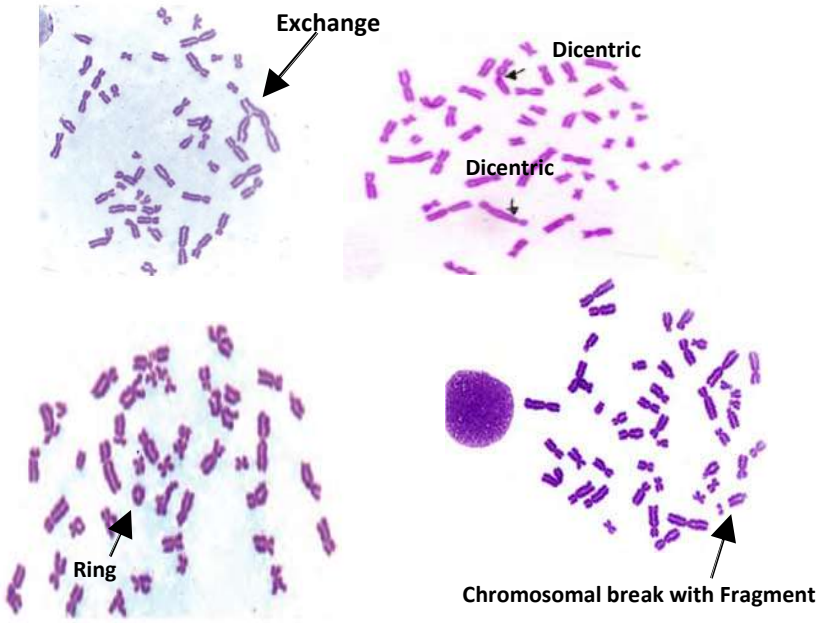
triflurothymidine (TFT). Thymidine kinase proficient cells are sensitive to TFT, which causes inhibition of cellular metabolism and halts further cell division. Thus, mutant cells are able to proliferate in the presence of TFT, whereas normal cells which contain TK enzyme are not. TK mutants include normal growing and slow growing mutants. These are recognized as "large colony" and "small colony". Large colonies are considered indicative of chemicals inducing point and other small-scale mutations whereas small colonies are predictive of chemicals that induce chromosomal damage.

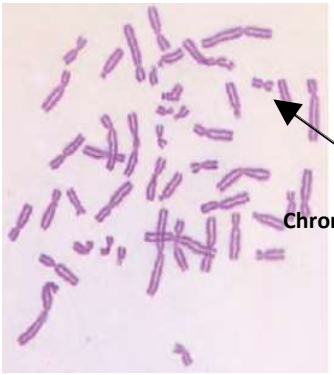
For Mouse Lymphoma Assay, the test protocol should include the conduct of tests with and without metabolic activation, with appropriate positive and negative controls, where the treatment with the test substance is for 3 to 4 hours. A continuous treatment without metabolic activation for approximately 24 hours should be conducted in case of a negative or equivocal result for both short treatments, with and without metabolic activation. A standard Mouse Lymphoma Assay should include (i) the incorporation of positive controls that induce mainly small colonies, and (ii) colony sizing for positive controls, solvent controls and at least one positive test compound concentration (should any exist), including the culture that gave the greatest mutant frequency.

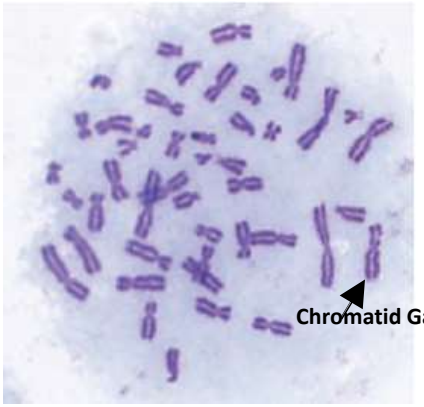
c) Test for Chromosomal Aberrations in Mammalian Cells In-vitro

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 clastogenic agents. The damage is scored by microscopic examination of chromosomes in mitotic metaphase cells.

Fig 2: Aberrated Chromosomes







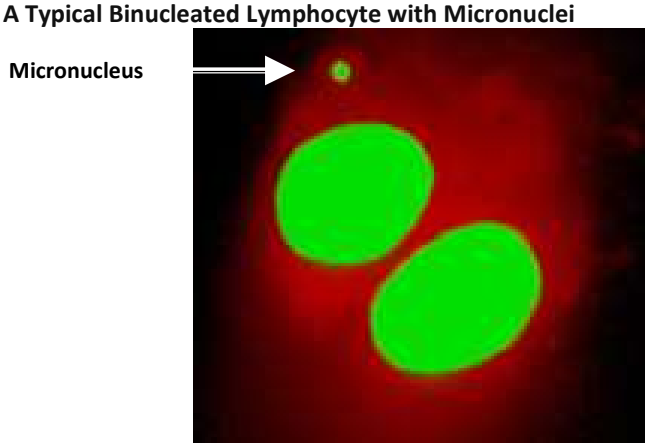
The cultured Chinese Hamster Ovary (CHO) cells, V79 or CHL cells or human peripheral blood lymphocytes are treated with chemicals in the presence or absence of metabolic activation. Depending on the phase of the cell cycle, chromosome mutations will manifest as chromosome-type aberrations (when they 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.

The maximum top concentration recommended is 1 mM or 0.5 mg/ml, whichever is lower, when not limited by solubility in solvent or culture medium or by cytotoxicity. At highest dose cytotoxicity should not exceed a reduction of about 50% in cell growth. Cells cultures are exposed to test item with and without metabolic activation at pre-determined intervals, treated with metaphase arresting agent, harvested, stained and metaphase are analyzed microscopically for the presences of chromosome aberrations. Treatment with the test articles should be for 3 to 6 hours with a sampling time approximately 1.5 normal cell cycles from the beginning of the treatment. A continuous treatment without metabolic activation up to the sampling time of approximately 1.5 normal cell cycles should be conducted in case of negative or equivocal results for both short treatments, with and without metabolic activation.

d) *In-vitro* Micronucleus Test

The micronucleus test is used for detection of damage to the chromosomes or the mitotic apparatus induced by chemicals. Micronuclei are small particles consisting of acentric fragments, or entire chromosomes that lag behind at anaphase of cell division. 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.

Fig 3: A Typical Binucleated Lymphocyte with Micronuclei

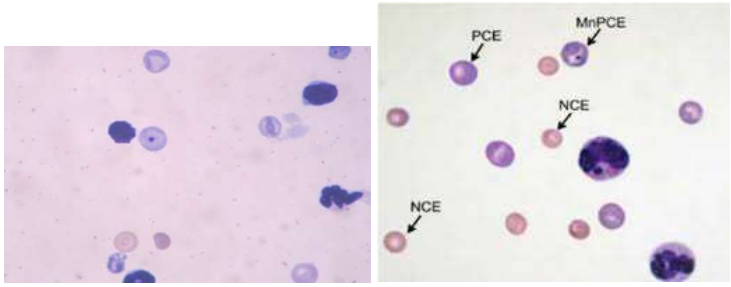


The test can be conducted using cultured primary human or other mammalian peripheral blood lymphocytes and a number of cell lines such as CHO, V79, and CHL etc. For dose selection and treatment same criteria is applied for *in-vitro* micronucleus test as for chromosomal aberration test, except that the sampling time is typically 1.5 to 2 normal cell cycles from the beginning of treatment to allow cells to complete mitosis and enter the next interphase. The scoring of micronuclei is generally conducted in the first division of cells after test substance exposure. Cytochalasin B can be used to block cytoplasm division/cytokinesis and generate binucleate cells during or after test substance exposure. This may be desirable, because it can be used to measure cell proliferation and allows the scoring of micronuclei in dividing cells only. This assay is more easily scored than the chromosome aberration assay thus requiring less time to make an assessment of genotoxic potential of a chemical.

e) *In-vivo* Micronucleus Assay

Measurement of micronucleated polychromatic erythrocytes in bone marrow cells *in-vivo* is one of the acceptable tests by regulatory agencies for the detection of clastogens. Either rats or mice are generally used for bone marrow micronucleus test. Micronuclei can also be measured in immature (e.g., polychromatic) erythrocytes in peripheral blood in the mouse, or in the newly formed reticulocytes in rat blood. When a bone marrow erythroblast develops into an immature erythrocyte (polychromatic erythrocyte, or reticulocyte) and then migrates into the peripheral blood, the main nucleus is extruded. Subsequently, any micronuclei that have been formed may remain behind in the cytoplasm. Thus, detection of micronuclei is facilitated in erythrocytes because they lack a main nucleus.

Fig 4: Bone Marrow Micronucleus



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.

Dose levels selected for the study should cover a range from no or little toxicity to the maximum tolerated dose. If toxicity is not the limiting factor, 2000 mg/kg is the limit dose for short term studies (<14 days) and 1000 mg/kg is the limit dose for treatment period of ≥ 14 days.

f) *In-vivo* Chromosomal Aberration Test

The mammalian bone marrow chromosomal aberration test identifies substances that induce structural chromosomal aberrations in bone marrow cells. While rodents are usually used, other species can be used as per scientific rationale. General principle of *in-vivo* chromosomal aberration test is same as *in-vitro* chromosomal aberration test. Dose selection criteria is same as for *in-vivo* micronucleus test.

G. Acceptance Criteria of Genotoxicity Assays

Test guidelines for each assay specify the recommendations for individual assay acceptability. General points common to almost all genotoxicity assays are as follows:

- Concurrent negative control should show spontaneous mutants/genotoxic events within the range of laboratory historical negative control database, and/or published reference range
- Concurrent positive controls induce responses within range of historical control data of laboratory's historical positive control data base, and produce a statistically significant increase compared with the concurrent negative control;
- The tests are conducted in accordance with appropriate test protocols as per guidance documents, including
  - For *in-vitro* assays, all experimental conditions (based on the recommended treatment times and including the absence and presence of metabolic activation) are tested unless one resulted in clear positive results;
  - Adequate numbers of animals/cells were treated and carried through the experiment or scored
  - Adequate number of doses/concentrations covering the appropriate dose/concentration range is analyzable.

- Criteria for the selection of highest dose/concentration are consistent with those described in the individual test guidelines.

H. Evaluation Criteria of Genotoxicity Assays

For both *in-vitro* and *in-vivo* genotoxicity assays a test article is considered positive if it meets all the criteria mentioned below in at least one experimental condition:

- At least one of the data points exhibits a statistically significant increase compared to the concurrent negative control
- The increase in genotoxic response is dose dependent and reproducible
- The result is outside the historical negative control data
- For Mouse lymphoma assay for positive results, the mutant frequency should be above the Global Evaluation Factor defined in the test guideline.

If none of the above criteria is met, then test substance is classified as negative for genotoxic potential.

Generally, meeting the requirement of test guidelines, it is possible to classify the test substance as positive or negative. In certain cases, when the response in neither clearly positive nor negative, it requires, further experimentation with modification of test methods (e.g. change in concentration spacing, change in S9 concentration or origin, change in treatment duration or sampling time etc.). However, if even after repetition and expert judgment, it is not possible to classify the compound as either positive or negative, then it is concluded as equivocal.

I. Interpretation of Results

The objective of the genotoxicity testing procedures is to establish with reasonable certainty whether a substance possesses genotoxic potential or not. Following from this is a second and quite separate issue, what 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 genotoxic hazard is of an acceptable low order (although it may be considered evidence of absence of carcinogenic potential). If all results indicate that the compound has genotoxic 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.

While evaluating the genotoxic potential of the compound a number of factors should be considered:

- Genetic endpoints (e.g., gene mutations, structural or numerical chromosomal aberrations) detected by the test systems.
- Sensitivity and predictive value of the test systems for various classes of chemical compounds. Based on literature predicted sensitivity and specificity of various assays is: Bacterial reversion (Ames) 60 and 77 %, Chromosome aberrations 70 and 55 %, Mammalian mutation 81 and 48% respectively.
- Number of different test systems used for detecting each genetic endpoint e.g. Bacterial reverse mutation test may not be suitable for testing a peptide that degrade and can supplement media with required amino acids. In those cases, mammalian gene mutation test may be more relevant.
- Consistency and reproducibility of the results obtained in different test systems and different species. Weak/equivocal response that are not reproducible is generally considered not biologically relevant.
- Dose-response relationship
- Biological significance of the results. A small but statistical increase in apparent toxicity may not be biologically relevant.
- Experimental conditions in which positive results are being observed. Any positive result at only highly toxic doses/concentrations or under conditions which do not occur *in-vivo* (e.g. high pH, osmolality and precipitation) are not considered biologically relevant. Similarly, a positive result in an *in-vivo* test deserves more weight than a negative result *in-vitro*, as *in-vitro* metabolic activation might be in-adequate to generate genotoxic metabolite.

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. Negative result in *in-vivo* condition might be due to inadequate exposure of target tissue. Hence, to show adequate exposure at target tissue either in terms of drug concentration or cytotoxicity is one of the key aspects for *in-vivo* tests.

J. Follow Up Tests

In general, the standard genotoxicity test battery is adequate for evaluation of genotoxicity of a compound. However, on rare occasions,

contradictory results in different assay and insufficient weight of evidence to indicate non –relevance of one genotoxicity assay, necessitate further testing. Such additional testing may provide mechanistic information for chronic rodent carcinogenicity bioassay. The choice of follow-up test should be guided by the spectrum of genotoxic events observed in the *in-vitro* studies as well as knowledge of the bioavailability, distribution, metabolism and target organ specificity of the substance. Typically, a bone marrow micronucleus or clastogenicity test is conducted. However, if there are indications that point to a more appropriate assay, then this assay should be conducted instead (e.g. mutagenicity study with transgenic animals and/or comet assay in potential target tissues). Other methods include (but are not limited to) Expanded Simple Tandem Repeat (ESTR) assay, chromosomal assays (including those using fluorescence in situ hybridization), liver Unscheduled DNA synthesis covalent binding and DNA adduct analysis. These tests will increase both the sensitivity and specificity of the existing test protocols.

For substances that give positive results for mutagenic effects in somatic cells *in-vivo*, their potential to affect germ cells should be considered. If there is toxicokinetic or toxicodynamic evidence that germ cells are actually exposed to the somatic mutagen or its bioactive metabolites, it is reasonable to assume that the substance may also pose a mutagenic hazard to germ cells and thus a risk to future generations. There are a number of tests available, e.g. clastogenicity in rodent spermatogonial cells, dominant lethal test and mouse heritable translocation assay.

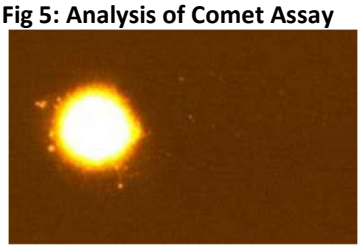
The ICH guidelines do not exclude the new methods and encourage development of new systems and their use, when strong scientific justifications support the findings.

One of the commonly used alternative/follow-up test is Comet Assay to assess the DNA damage. OECD guidance document is also recently added for this assay.

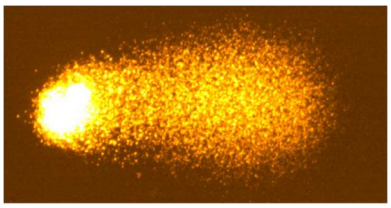
The comet assay identifies substances that induce primary DNA damage. An alternate name is the alkaline single-cell gel electrophoresis assay. Under alkaline conditions (> pH 13), the comet assay can detect single and double strand breaks in eukaryotic cells, resulting, for example, from direct interactions with DNA alkali labile sites, or as a consequence of transient DNA strand discontinuities resulting from DNA excision repair. These strand breaks may be: 1) repaired, resulting in no persistent effect; 2) lethal to the cell; or 3) fixed as a mutation resulting in a permanent heritable change.

Therefore, the alkaline comet assay detects primary DNA strand breaks that do not always lead to gene mutations and/or chromosomal aberrations. The assay involves image analysis of the migration pattern caused by fragmented DNA in an agarose gel. The more fragmented the DNA is, the more the DNA fragments migrate, forming a structure that resembles the tail of a comet. Damage quantification is then based on the calculation of the 'head' (the actual cell) and the 'tail' (the migrated fragments).

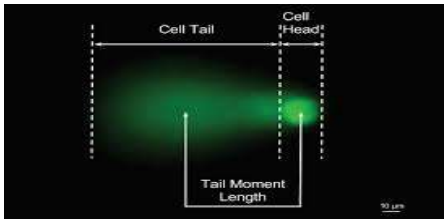
Fig 5: Analysis of Comet Assay



A: Control



B: Treated



C: Analysis

The comet assay is amenable for both *in-vitro* (in any cell type) and *in-vivo* or ex-*vivo,* any species and in any target tissue. 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). As a result, the comet assay can be incorporated in any routine toxicology experiments, which can add value without adding any extra animals. The Comet assay is a promising tool because it is rapid, simple to perform, and requires only a small amount of test substance.

Using the comet assay, 208 chemicals selected from the IARC monographs and US NTP carcinogenicity database were investigated. 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. 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.

CONCLUSIONS

Genotoxicity studies are important to be conducted as this is a major health hazard to human and economically important animals. Any agent that can interact with the DNA thus causing mutations and damaging its structure, may lead to cancer or other heritable diseases. Therefore, it is very important to conduct genotoxicity studies in order to avoid the potential damage that can be caused by a genotoxic material. Identification of genotoxic agents before human exposure helps us understand the mechanism of the mutation and genotoxicity thereby paving us way to avoid such deleterious effects.

A tiered approach is applied in regulatory mutagenicity testing. In the first step, *in-vitro* assays with a high sensitivity are used to identify test compounds that have high intrinsic genotoxic activity. In the second step, specific *in-vivo* tests are performed to determine the relevance of *in-vitro* results for the *in-vivo* conditions. These *in-vivo* genotoxicity studies are also important as some agents can only be identified after metabolic activation in *in-vivo* system. Compared to regulatory carcinogenicity testing, genotoxicity testing is relatively cheaper and faster. Compounds without genotoxic liability can be proceeded directly for clinical trials. The carcinogenic potential is assessed later in the full developmental phase of drug development; however, the regulatory test strategy consists of a battery of core and ancillary tests for identifying the three forms of genotoxicity (i.e., gene mutations, clastogenicity, and aneugenicity), which cannot be detected by one single test. It is important to emphasize that data generated from these studies should be evaluated and interpreted carefully along with the weight of evidence, to appropriately characterize the compound.

Questions

1. Define Genetic Toxicology?
2. What are the two options for battery of genotoxicity?
3. Why is it important to conduct a battery of genotoxicity studies?
4. Which bacterial strains should be used in Ames test?
5. What is the importance of using S9 in in-vitro genotoxicity studies?
6. In micronucleus test what type of genotoxic agents can be identified?
7. What are the supplementary approached to confirm the equivocal results in standard battery of test?
8. Why it is important to use negative and positive controls in genotoxicity assays?
9. Why it is important to do sizing of the colonies in mammalian gene mutation test?
10. What is the difference between mutagens, clastogens and aneugens?

---------End of the Document----------

ToxGurukul Foundation

ToxGurukul Foundation is a registered non-profit organization for professionals in the field of toxicology who are in search of a platform to learn and share the vast knowledge in this area. This syndicate belongs to independent professionals from different backgrounds of toxicology who share their knowledge to un-puzzle the Rubik's cube that each face in their daily work routine.

Website: ***www.toxgurukul.org***
Email: ***toxgurukul.india@gmail.com***

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Regd. Office: Fl. No.10, New Ajanta Avenue, Building-4, Wing- A1, S. No. 135/136 Part, Kothrud, Pune-411038
Corporate Identity Number: U80904PN2019NPL182886
Email Id: toxgurukul.india@gmail.com