

Casarett & Doull's Essentials of Toxicology, 2e >

Chapter 9. Genetic Toxicology

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Key Points

- Genetic toxicology assesses the effects of chemical and physical agents on the hereditary material (DNA) and on the genetic processes of living cells.
- *Oncogenes* are genes that stimulate the transformation of normal cells into cancer cells.
- Genetic toxicology assays serve to identify mutagens for purposes of hazard identification, and to characterize dose–response relationships and mutagenic mechanisms.
- A broad range of short-term assays for genetic toxicology serve to identify many mutagens and address the relationship between mutagens and cancer-causing agents.

Genetic Toxicology: Introduction

Genetic toxicology assesses the effects of chemical and physical agents on both DNA and on the genetic processes of living cells. This chapter addresses the assays for qualitative and quantitative assessment of cellular changes induced by chemical and physical agents, the underlying molecular mechanisms for these changes, and how such information can be incorporated in risk assessments.

Health Impact of Genetic Alterations

Somatic Cells

Oncogenes are genes that stimulate the transformation of normal cells into cancer cells. They originate when genes called proto-oncogenes, involved in normal cellular growth and development, are genetically altered. Mutational alteration of proto-oncogenes can lead to overexpression of their growth-stimulating activity, whereas mutations that inactivate tumor-suppressor genes, which normally restrain cellular proliferation, free cells from their inhibitory influence.

The action of oncogenes is genetically dominant in that a single active oncogene is expressed even though its normal allele is present in the same cell. Among chromosomal alterations that activate proto-oncogenes, translocations are especially prevalent. A translocation can activate a proto-oncogene by moving it to a new chromosomal location with a more active promoter, where its expression is enhanced.

Unlike oncogenes, the cancer-causing alleles that arise from tumor-suppressor genes are typically recessive in that they are not expressed when they are heterozygous. Gene mutations in a tumor-suppressor gene on chromosome 17, called *P53*, occur in many different human cancers, and molecular characterization of *P53* mutations has linked specific human cancers to mutagen exposures.

Six acquired characteristics are essential for the formation of all tumors irrespective of tumor type and species. These include: (1) self-sufficiency in growth signals, (2) insensitivity to antigrowth signals, (3) evasion of apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis. It seems probable that there is no specific order for obtaining these characteristics.

Gene mutations, chromosome aberrations (morphologic abnormality), and aneuploidy (abnormal number of chromosomes) are all implicated in the development of cancer. Many mutagens and clastogens (chromosome-breaking agents) contribute to carcinogenesis as initiators; however, mutagens, clastogens, and aneugens also may contribute to multiple genetic alterations.

Germ Cells

The relevance of gene mutations to health is evident from the many disorders often caused by base-pair substitutions or small deletions that are inherited as simple Mendelian characteristics. Many genetic disorders (e.g., cystic fibrosis, phenylketonuria, and Tay–Sachs disease) are caused by the expression of recessive mutations. These mutations are mainly inherited from previous generations and are expressed when an individual inherits the mutant gene from both parents.

Besides causing diseases that exhibit Mendelian inheritance, gene mutations undoubtedly contribute to human disease through the genetic component of disorders with a complex etiology such as heart disease, hypertension, and diabetes. Refined cytogenetic methods have led to the discovery of minor variations in chromosome structure that have no apparent effect. Nevertheless, other chromosome aberrations cause fetal death or serious abnormalities. Aneuploidy also contributes to fetal deaths and causes disorders such as Down syndrome. Much of the effect of chromosomal abnormalities occurs prenatally. Among the abnormalities, aneuploidy is the most common, followed by polyploidy. Structural aberrations constitute about 5 percent of the total. Most chromosomal anomalies detected in newborns arise de novo in the germ cells of the parents.

Cancer and Genetic Risk Assessments

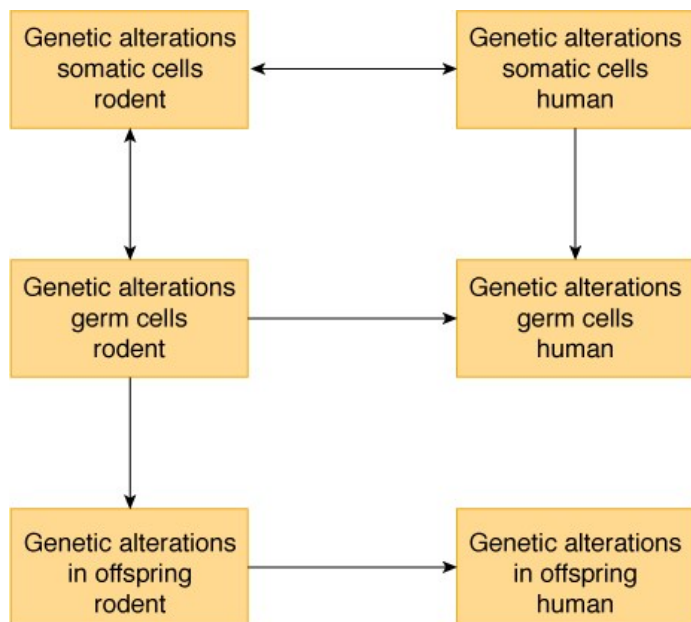
Cancer Risk Assessment

Cancer risk assessment involves investigation of sensitivity of different species and subpopulations to tumor induction by a chemical and development of a dose–response curve of mutations to a chemical.

Genetic Risk Assessment

To investigate genetic risk, the frequency of genetic alteration in human germ cells is estimated by extrapolation from data from rodent germ cells and somatic cells. For a complete estimate of genetic risk, it is necessary to obtain an estimate of the frequency of genetic alterations transmitted to the offspring (Figure 9–1).

Figure 9-1



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Parallelogram approach for genetic risk assessment. Data obtained for genetic alterations in rodent somatic and germ cells and human

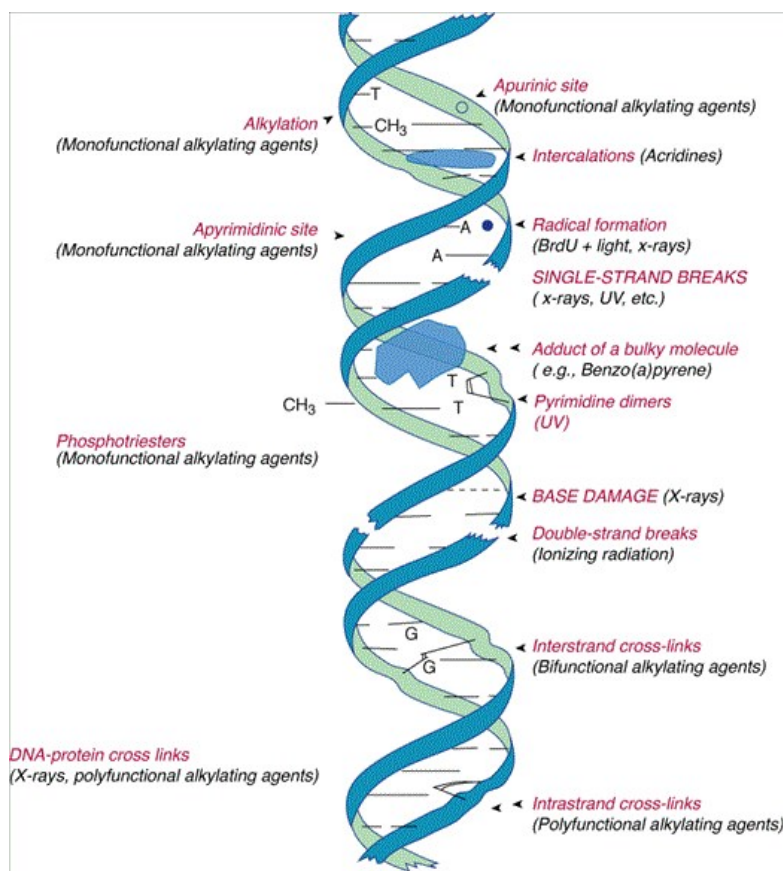
somatic cells are used to estimate the frequency of the same genetic alterations in human germ cells. The final step is to estimate the frequency of these genetic alterations that are transmitted to offspring.

Mechanisms of Induction of Genetic Alterations

DNA Damage

The types of DNA damage range from single- and double-strand breaks in the DNA backbone to cross-links between DNA bases and between DNA bases and proteins and chemical addition to the DNA bases (adducts) (Figure 9-2).

Figure 9-2



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Spectrum of DNA damage induced by physical and chemical agents.

Ionizing Radiations

Ionizing radiations, such as X-rays, gamma rays, and alpha particles, produce DNA single- and double-strand breaks and a broad range of base damages. Multiple damaged sites or cluster lesions appear to be more difficult to repair. These multiple lesions can be formed in DNA from the same radiation energy deposition event. The relative proportions of these different classes of DNA damage vary with type of radiation.

Ultraviolet Light

Ultraviolet light (a nonionizing radiation) induces two predominant lesions, cyclobutane pyrimidine dimers and 6,4-photoproducts. These lesions can be quantitated by chemical and immunologic methods.

Chemicals

Chemicals can produce base alterations either directly (DNA-reactive) as adducts or indirectly by intercalation of a chemical between the base pairs. Many electrophilic chemicals react with DNA, forming covalent addition products (adducts). Alkylated bases can also lead to base loss from DNA, which leaves an apurinic or apyrimidinic site, commonly called an AP site. The insertion of incorrect bases into AP sites causes mutations. Bulky DNA adducts are recognized by the cell in a similar way to UV damages and are repaired similarly.

Endogenous Agents

Endogenous agents are responsible for several hundred DNA damages per cell per day. The majority of these damages are altered DNA bases (e.g., 8-oxoguanine and thymine glycol) and AP sites. The cellular processes that can lead to DNA damage are the formation of reactive active oxygen species and deamination of cytosines and S-methylcytosines leading to uracils and thymines, respectively. The process of DNA replication itself is error-prone, and an incorrect base can be added by the polymerase.

DNA Repair

Two processes enable the cell to cope with the DNA damage that it sustains. With extensive damage, the cell can undergo apoptosis. If the damage is less severe, cells have developed a range of repair processes that return the DNA to its undamaged state (error-free repair) or to an improved but still altered state (error-prone repair). The basic principles underlying most repair processes are damage recognition, removal of damage (except for strand breaks or cleavage of pyrimidine dimers), repair DNA synthesis, and ligation.

Base Excision Repair

The major pathways by which DNA base damages are repaired involve removal of the damaged base. The resulting gap can be filled by a DNA polymerase, followed by ligation to the parental DNA. Sites of oxidative damage, either background or induced, are important substrates for base excision repair.

Nucleotide Excision Repair

The nucleotide excision repair (NER) system provides the cell's ability to remove bulky lesions from DNA. NER removes a damage-containing oligonucleotide from DNA by damage recognition, incision, excision, repair synthesis, and ligation. The DNA damage in actively transcribing genes, and specifically the transcribed strand, is preferentially and more rapidly repaired than the DNA damage in the rest of the genome. Thus, the cell protects the integrity of the transcription process.

Double-Strand Break Repair

Cell survival is seriously compromised by the presence of broken chromosomes. Unrepaired double-strand breaks trigger one or more DNA damage response systems to either check cell-cycle progression or induce apoptosis. There are two general pathways for repair of DNA double-strand breaks: homologous recombination and nonhomologous end-joining (NHEJ).

Homologous Recombination

The repair of double-strand breaks (and single-strand gaps) uses the following basic steps. The initial step is the production of a 3'-ended single-stranded tail by exonucleases or helicase activity. Through strand invasion, whereby the single-stranded tail invades an undamaged homologous DNA molecule, together with DNA synthesis, a so-called Holliday junction DNA complex is formed. By cleavage of this junction, two DNA molecules are produced (with or without a structural crossover), neither of which now contain a strand break.

Nonhomologous End-Joining

This type of recombination requires the production of double-strand breaks, recombination of DNA pieces, and subsequent religation. A major component of the NHEJ repair complex is a DNA-dependent protein kinase (DNA-PK). This protein probably functions to align the broken DNA ends to facilitate their ligation or to serve as a signal molecule for recruiting other repair proteins.

Mismatch Repair

DNA mismatch repair systems operate to repair mismatched bases. The principal steps are damage recognition by a specific protein that binds to the mismatch, stabilization of the binding by the addition of one or more proteins, cutting the DNA at a distance from the mismatch, excision past the mismatch, resynthesis, and ligation.

O⁶-Methylguanine-DNA Methyltransferase Repair

The enzyme O⁶-methylguanine-DNA methyltransferase (MGMT) protects cells against the toxic effects of simple alkylating agents by transferring the methyl group from O⁶-methylguanine in DNA to a [cysteine](#) residue in MGMT. The adducted base is reverted to a normal one by the enzyme, which is itself inactivated by the reaction.

Formation of Gene Mutations

Somatic Cells

Gene mutations, considered to be small DNA-sequence changes confined to a single gene, are substitutions, small additions, and small deletions. Base substitutions are the replacement of the correct nucleotide by an incorrect one; they can be further subdivided as transitions, where the change is purine for purine or pyrimidine for pyrimidine, and transversions where the change is purine for pyrimidine or vice versa. Frameshift mutations are the addition or deletion of one or a few base pairs (not in multiples of 3) in protein-coding regions.

The great majority of so-called spontaneous (background) mutations arise from *replication* of an altered template. These DNA alterations are either the result of oxidative damage or produced from the deamination of 5-methyl cytosine to thymine at CpG sites resulting in G:C → A:T transitions.

Gene mutations produced by a majority of chemicals and nonionizing radiations are base substitutions, frameshifts, and small deletions. Of these mutations, most are produced by errors of DNA *replication* on a damaged template. The relative mutation frequency will be the outcome of the race between repair and replication, that is, the more repair that takes place prior to replication, the lower the mutation frequency for a given amount of induced DNA damage. Significant regulators of the race are cell-cycle checkpoint genes (e.g., *P53*) because if the cell is checked from entering the S phase at a G₁/S checkpoint, then more repair can take place prior to the cell starting to replicate its DNA.

Germ Cells

The mechanism of production of gene mutations in germ cells is basically the same as in somatic cells. Ionizing radiations produce mainly deletions via errors of DNA repair; the majority of chemicals induce base substitutions, frameshifts, and small deletions by errors of DNA replication.

An important consideration for assessing gene mutations induced by chemicals in germ cells is the relationship between exposure and the timing of DNA replication (i.e., if there is damage, is it able to be repaired before replication?). The spermatogonial stem cell is the major contributor to genetic risk assessment because it is present generally throughout the reproductive lifetime of an individual. Each time a spermatogonial stem cell divides, it produces a differentiating spermatogonium and a stem cell. This stem cell can accumulate genetic damage from chronic exposures.

In oogenesis, the primary oocyte arrests prior to birth, and there is no further S phase until the zygote. For this reason, the oocyte is resistant to the induction of gene mutations by most chemicals.

Formation of Chromosomal Alterations

Somatic Cells

Structural Chromosome Aberrations

Sister chromatid exchanges (SCEs; the apparently reciprocal exchange between the sister chromatids of a single chromosome) and gene mutations are common. In particular, damaged DNA serves as the substrate leading to chromosomal aberrations. The DNA repair errors that lead to the formation of

chromosome aberrations following ionizing radiation exposure arise from misligation of double-strand breaks or interaction of coincidentally repairing regions during NER of damaged bases. Incorrect rejoining of chromosomal pieces during repair leads to chromosomal exchanges within and between chromosomes. Failure to rejoin double-strand breaks or to complete repair of other types of DNA damage leads to terminal deletions.

The failure to incorporate an acentric fragment into a daughter nucleus at anaphase/telophase, or the failure of a whole chromosome to segregate to the cellular poles at anaphase, can result in the formation of a micronucleus that resides in the cytoplasm. Errors of DNA replication on a damaged template can lead to a variety of chromosomal alterations. The majority of these involve deletion or exchanges of individual chromatids but some can involve both chromatids.

Numerical Chromosome Changes

Numerical changes (e.g., monosomies, trisomies, and ploidy changes) can arise from errors in chromosomal segregation due to any of the numerous possible impairments of mitotic control processes. Alteration of various cellular components can result in failure to segregate the sister chromatids to separate daughter cells or in failure to segregate a chromosome to either pole.

Sister Chromatid Exchange

SCEs are produced during S phase and are presumed to be a consequence of errors in the replication process.

Germ Cells

The formation of chromosomal alterations in germ cells is basically the same as that for somatic cells, namely, via misrepair for ionizing radiations and radiomimetic chemicals for treatments in G₁ and G₂, and by errors of replication for all radiations and chemicals for DNA damage present during the S phase.

The types of aberrations formed in germ cells are the same as those formed in somatic cells. The specific segregation of chromosomes during meiosis influences the probability of recovery of an aberration, particularly a reciprocal translocation, in the offspring of a treated parent.

Assays for Detecting Genetic Alterations

Introduction to Assay Design

Genetic toxicology assays serve two interrelated but distinct purposes in the toxicologic evaluation of chemicals: (1) identifying mutagens for purposes of hazard identification and (2) characterizing dose–response relationships and mutagenic mechanisms.

Table 9–1 lists many of the assays employed in genetic toxicology. Some assays for gene mutations detect forward mutations, whereas others detect reversion. Forward mutations are genetic alterations in a wild-type gene and are detected by a change in phenotype caused by the alteration or loss of gene function. In contrast, a back mutation or reversion is a mutation that restores gene function in a mutant and thereby brings about a return to the wild-type phenotype. The simplest gene mutation assays rely on selection techniques to detect mutations. By imposing experimental conditions under which only cells or organisms that have undergone mutation can grow, selection techniques greatly facilitate the identification of rare cells that have experienced mutation among the many cells that have not.

Table 9–1 Overview of genetic toxicology assays.

- | |
|---|
| <p>I. DNA damage and repair assays</p> <p>A. Direct detection of DNA damage</p> <ul style="list-style-type: none"> • Alkaline elution assays for DNA strand breakage • Comet assay for DNA strand breakage • Assays for chemical adducts in DNA <p>B. Bacterial assays for DNA damage</p> <ul style="list-style-type: none"> • Differential killing of repair-deficient and wild-type strains |
|---|

- Induction of the SOS system by DNA damage
- C. Assays for repairable DNA damage in mammalian cells
- Unscheduled DNA synthesis (UDS) in rat hepatocytes
- UDS in rodent hepatocytes in vivo

II. Prokaryote gene mutation assays

A. Bacterial reverse mutation assays

- *Salmonella*/mammalian microsome assay (Ames test)
- *E. coli* WP2 **tryptophan** reversion assay
- *Salmonella* specific base-pair substitution assay (Ames-II assay)
- *E. coli lacZ* specific reversion assay

B. Bacterial forward mutation assays

- *E. coli lacI* assay
- Resistance to toxic metabolites or analogs in *Salmonella*

III. Assays in nonmammalian eukaryotes

A. Fungal assays

- Forward mutations, reversion, and small deletions
- Mitotic crossing over and gene conversion in yeast
- Mitotic aneuploidy: chromosome loss or gain in yeast
- Meiotic nondisjunction in yeast or *Neurospora*

B. Plant assays

- Gene mutations affecting **chlorophyll** in seedlings or *waxy* in pollen
- Tradescantia stamen hair color mutations
- Chromosome aberrations or micronuclei in mitotic or meiotic cells
- Aneuploidy detected by pigmentation or cytogenetics

C. *Drosophila* assays

- Sex linked recessive lethal test in germ cells
- Heritable translocation assays
- Sex chromosome loss tests for aneuploidy
- Induction of mitotic recombination in eyes or wings

IV. Mammalian gene mutation assays

A. In vitro assays for forward mutations

- *tk* mutations in mouse lymphoma or human cells
- *hprt* or *xprt* mutations in Chinese hamster or human cells

B. In vivo assays for gene mutations in somatic cells

- Mouse spot test (somatic cell specific locus test)
- *hprt* mutations (6-thioguanine-resistance) in rodent lymphocytes

C. Transgenic assays

- Mutations in the bacterial *lacI* gene in “Big Blue[®]” mice and rats
- Mutations in the bacterial *lacZ* gene in the “Muta[™] Mouse”
- Mutations in the phage *cII* gene in *lacI* or *lacZ* transgenic mice
- Point mutations and deletions in the *lacZ* plasmid mouse

- Point mutations and deletions in *delta gpt* mice and rats

V. Mammalian cytogenetic assays

A. Chromosome aberrations

- Metaphase analysis in cultured Chinese hamster or human cells
- Metaphase analysis of rodent bone marrow or lymphocytes in vivo

B. Micronuclei

- Cytokinesis-block micronucleus assay in human lymphocytes
- Micronucleus assay in mammalian cell lines
- In vivo micronucleus assay in rodent bone marrow or blood

C. Sister chromatid exchange

- SCE in human cells or Chinese hamster cells
- SCE in rodent tissues, especially bone marrow

D. Aneuploidy in mitotic cells

- Mitotic disturbance seen by staining spindles and chromosomes
- Hyperploidy detected by chromosome counting
- Chromosome gain or loss in cells with intact cytoplasm
- Micronucleus assay with centromere labeling
- Hyperploid cells in vivo in mouse bone marrow
- Mouse bone marrow micronucleus assay with centromere labeling

VI. Germ cell mutagenesis

A. Measurement of DNA damage

- Molecular dosimetry based on mutagen adducts
- UDS in rodent germ cells
- Alkaline elution assays for DNA strand breaks in rodent testes

B. Gene mutations

- Mouse specific-locus test for gene mutations and deletions
- Mouse electrophoretic specific-locus test
- Dominant mutations causing mouse skeletal defects or cataracts
- Mouse tandem-repeat loci analysis

C. Chromosomal aberrations

- Cytogenetic analysis in oocytes, spermatogonia, or spermatocytes
- Micronuclei in mouse spermatids
- Mouse heritable translocation test

D. Dominant lethal mutations

- Mouse or rat dominant lethal assay

E. Aneuploidy

- Cytogenetic analysis for aneuploidy arising by nondisjunction
- Sex chromosome loss test for nondisjunction or breakage
- Micronucleus assay in spermatids with centromere labeling
- FISH with probes for specific chromosomes in sperm

Studying mutagenesis in intact animals requires more complex assays, which range from inexpensive short-term tests that can be performed in a few

days to complicated assays for mutations in mammalian germ cells. Typically, there remains a gradation in which an increase in relevance for human risk entails more elaborate and costly tests.

Many compounds that are not themselves mutagenic or carcinogenic can be activated into mutagens and carcinogens by mammalian metabolism. Such compounds are called promutagens and procarcinogens. The most widely used metabolic activation system in microbial and cell culture assays is a postmitochondrial supernatant from a rat liver homogenate, along with appropriate buffers and cofactors. Most of the short-term assays in [Table 9-1](#) require exogenous metabolic activation to detect promutagens. Exceptions are those in intact mammals.

Despite their usefulness, *in vitro* metabolic activation systems cannot mimic mammalian metabolism perfectly. There are differences among tissues in reactions that activate or inactivate foreign compounds, and organisms of the normal flora of the gut can contribute to metabolism in intact mammals.

DNA Damage and Repair Assays

Some assays measure DNA damage itself rather than mutational consequences of DNA damage. They may do so directly, through such indicators as chemical adducts or strand breaks in DNA, or indirectly, through measurement of biological repair processes. Adducts in DNA can be detected by ³²P-postlabeling, immunologic methods using antibodies against specific adducts, or fluorometric methods in the case of such fluorescent compounds.

A rapid method of measuring DNA damage is the comet assay. In this assay, cells are incorporated into agarose on slides, lysed so as to liberate their DNA, and subjected to electrophoresis. The DNA is stained with a fluorescent dye for observation and image analysis. Because broken DNA fragments migrate more quickly than larger pieces of DNA, a blur of fragments (a “comet”) is observed when the DNA is extensively damaged. The extent of DNA damage can be estimated from the length and other attributes of the comet tail. The comet assay appears to be a sensitive indicator of DNA damage with broad applicability among diverse species, including plants, worms, mollusks, fish, and amphibians.

The occurrence of DNA repair can serve as a readily measured indicator of DNA damage. The most common excision repair assay in mammalian cells is an assay for unscheduled DNA synthesis (UDS). The occurrence of UDS indicates that the DNA had been damaged.

Gene Mutations in Prokaryotes

The most common means of detecting mutations in microorganisms is selecting for reversion in strains that have a specific nutritional requirement differing from wild-type members of the species; such strains are called auxotrophs. In the Ames assay, one measures the frequency of histidine-independent bacteria that arise in a histidine-requiring strain in the presence or absence of the chemical being tested. Auxotrophic (nutrient-deficient) bacteria are treated with the chemical of interest and plated on medium that is deficient in histidine; if the colony survives, it must have a reversion mutation that allows it to survive without exogenous histidine.

The development of specific reversion assays of histidine mutations in *Salmonella* strains and of *lacZ* mutations in *Escherichia coli* has made the identification of specific base-pair substitutions more straightforward.

Genetic Alterations in Nonmammalian Eukaryotes

Gene Mutations and Chromosome Aberrations

The fruit fly, *Drosophila*, has long occupied a prominent place in genetic research because of the sex-linked recessive lethal (SLRL) test. The SLRL test permits the detection of recessive lethal mutations at 600 to 800 different loci on the X chromosome by screening for the presence or absence of wild-type males in the offspring of specifically designed crosses. A significant increase over the frequency of spontaneous SLRLs in the lineages derived from treated males indicates mutagenesis. The SLRL test yields information about mutagenesis in germ cells, which is lacking in microbial and cell culture systems.

Genetic and cytogenetic assays in plants continue to find use in special applications, such as in situ monitoring for mutagens and exploration of the metabolism of promutagens by agricultural plants. In situ monitoring entails looking for evidence of mutagenesis in organisms that are grown in the environment of interest.

Mitotic Recombination

Assays in nonmammalian eukaryotes are important for the study of induced recombination. Recombinogenic effects in yeast have long been used as a general indicator of genetic damage. The best characterized assays for recombinogens are those that detect mitotic crossing over and mitotic gene conversion in the yeast *Saccharomyces cerevisiae*.

Gene Mutations in Mammals

Gene Mutations In Vitro

Mutagenicity assays in cultured mammalian cells have some of the same advantages as microbial assays with respect to speed and cost, and they follow quite similar approaches. The most widely used assays for gene mutations in mammalian cells detect forward mutations that confer resistance to a toxic chemical.

Gene Mutations In Vivo

In vivo assays involve treating intact animals and analyzing genetic effects in appropriate tissues. Mutations may be detected either in somatic cells or in germ cells.

The mouse spot test is a traditional genetic assay for gene mutations in somatic cells. Visible spots of altered phenotype in mice heterozygous for coat color genes indicate mutations in the progenitor cells of the altered regions.

Mutation assays also provide information on mechanisms of mutagenesis. Base-pair substitutions and large deletions can be differentiated through the use of probes for the target gene and Southern blotting, in that base substitutions are too subtle to be detectable on the blots. Gene mutations have been characterized at the molecular level by DNA-sequence analysis both in transgenic rodents and in endogenous mammalian genes.

Transgenic Assays

Transgenic animals are products of DNA technology in which the animal contains foreign DNA sequences that have been added to the genome and are transmitted through the germ line. The foreign DNA is therefore represented in all the somatic cells of the animal.

Mice that carry *lac* genes from *E. coli* use either *lacI* or *lacZ* as a target for mutagenesis. After mutagenic treatment of the transgenic animals, the *lac* genes are recovered from the animal, packaged into phage λ , and transferred to *E. coli* for mutational analysis. Mutant plaques are identified on the basis of phenotype, and mutant frequencies can be calculated for different tissues of the treated animals.

Mammalian Cytogenetic Assays

Chromosome Aberrations

Genetic assays without DNA sequencing are indirect, in that one observes a phenotype and reaches conclusions about genes. In contrast, cytogenetic assays use microscopy for direct observation of the effect of interest. In conventional cytogenetics, metaphase analysis is used to detect chromosomal anomalies. Cells should be treated during a sensitive period of the cell cycle (typically S), and aberrations should be analyzed at the first mitotic division after treatment. Examples of chromosome aberrations are shown in [Figure 9-3](#).

Figure 9-3



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Chromosome aberrations induced by x-rays in Chinese hamster ovary (CHO) cells.A. A chromatid deletion (

▶). B. A chromatid exchange called a triradial (

▶). C. A small interstitial deletion (

▶) that resulted from chromosome breakage. D. A metaphase with more than one aberration: a centric ring plus an acentric fragment (

▶) and a dicentric chromosome plus an acentric fragment (→).

It is essential that sufficient cells be analyzed because a negative result in a small sample is equivocal and inconclusive. Results should be recorded for specific classes of aberrations, not just as an overall index of aberrations per cell.

In interpreting results on the induction of chromosome aberrations in cell cultures, questionable positive results have been found at highly cytotoxic doses, high osmolality, and pH extremes. Although excessively high doses may lead to artifactual positive responses, the failure to test to sufficiently high doses also undermines the utility of a test; therefore, testing should be conducted at an intermediate dose and extended to a dose at which some cytotoxicity is observed.

In vivo assays for chromosome aberrations involve treating intact animals and later collecting cells for cytogenetic analysis. The main advantage of in vivo assays is that they include mammalian metabolism, DNA repair, and pharmacodynamics. The target is a tissue from which large numbers of dividing cells are easily prepared for analysis such as bone marrow.

In interphase cell analysis by fluorescence in situ hybridization (FISH), a nucleic acid probe is hybridized to complementary sequences in chromosomal

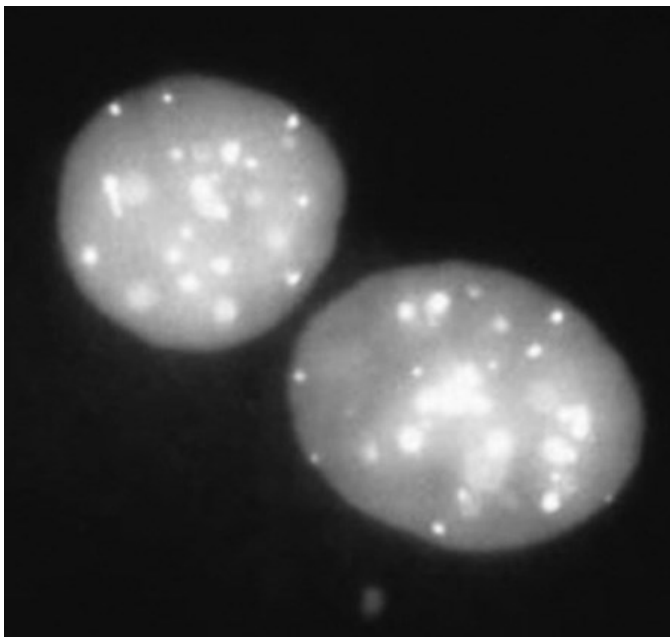
DNA. The probe is labeled with a fluorescent dye so that the chromosomal location to which it binds is visible by fluorescence microscopy; often, probes are used that cover the whole chromosome, called “chromosome painting.”

Chromosome painting facilitates cytogenetic analysis, because aberrations are easily detected by the number of fluorescent regions in a painted metaphase. FISH permits the scoring of stable aberrations, such as translocations and insertions, which are not readily detected in traditional metaphase analysis of unbanded chromosomes.

Micronuclei

Micronuclei are membrane-bounded structures that contain chromosomal fragments, or sometimes whole chromosomes, that were not incorporated into a daughter nucleus at mitosis. Micronuclei usually represent acentric chromosomal fragments, and they are commonly used as simple indicators of chromosomal damage. Micronuclei in a binucleate human lymphocyte are shown in [Figure 9-4](#).

Figure 9-4



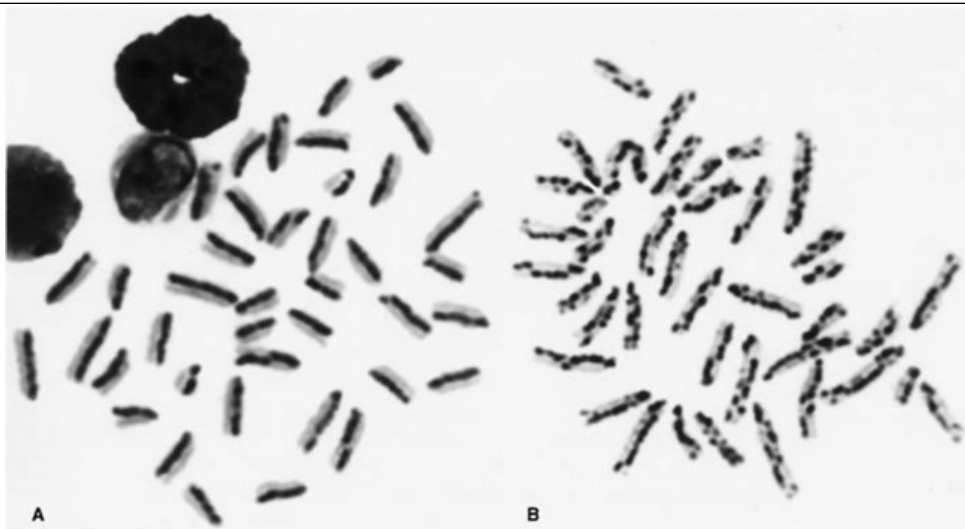
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Micronucleus in a human lymphocyte. The cytochalasin B method was used to inhibit cytokinesis that resulted in a binucleate nucleus. The micronucleus resulted from failure of an acentric chromosome fragment or a whole chromosome being included in a daughter nucleus following cell division. (Image courtesy of James Allen, Jill Barnes, and Barbara Collins.)

Sister Chromatid Exchange

SCE, in which apparently reciprocal segments have been exchanged between the two chromatids of a chromosome, is visible cytologically through differential staining of chromatids ([Figure 9-5](#)). SCE assays are general indicators of mutagen exposure, rather than measures of a mutagenic effect.

Figure 9-5



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Sister chromatid exchanges (SCEs) in human lymphocytes. A. SCEs in untreated cell. B. SCEs in cell exposed to ethyl carbamate. The treatment results in a very large increase in the number of SCEs. (Image courtesy of James Allen and Barbara Collins.)

Aneuploidy

Assays for aneuploidy include chromosome counting, the detection of micronuclei that contain kinetochores, and the observation of abnormal spindles or spindle–chromosome associations in cells in which spindles and chromosomes have been differentially stained.

The presence of the spindle attachment region of a chromosome (kinetochore) in a micronucleus can indicate that it contains a whole chromosome. Aneuploidy may therefore be detected by means of antikinetochore antibodies with a fluorescent label or FISH with a probe for centromere-specific DNA. Frequencies of micronuclei ascribable to aneuploidy and to clastogenic effects may therefore be determined concurrently by tabulating micronuclei with and without kinetochores.

Germ Cell Mutagenesis

Gene Mutations

Mammalian germ cell assays provide the best basis for assessing risks to human germ cells. Mammalian assays permit the measurement of mutagenesis at different germ cell stages. Late stages of spermatogenesis are often found to be sensitive to mutagenesis, but spermatocytes, spermatids, and spermatozoa are transitory. Mutagenesis in stem cell spermatogonia and resting oocytes is of special interest in genetic risk assessment because of the persistence of these stages throughout reproductive life.

Chromosomal Alterations

Knowledge of the induction of chromosome aberrations in germ cells is important for assessing risks to future generations. A germ cell micronucleus assay has been developed, in which chromosomal damage induced in meiosis is measured by observation of rodent spermatids. Aneuploidy originating in mammalian germ cells may be detected cytologically through chromosome counting for hyperploidy or genetically in the mouse sex-chromosome loss test.

Besides cytologic observation, indirect evidence for chromosome aberrations is obtained in the mouse heritable translocation assay, which measures reduced fertility in the offspring of treated males. This presumptive evidence of chromosomal rearrangements can be confirmed through cytogenetic analysis.

Dominant Lethal Mutations

The mouse or rat dominant lethal assay offers an extensive database on the induction of genetic damage in mammalian germ cells. Commonly, males are treated on an acute or subchronic basis with the agent of interest and then mated with virgin females. The females are killed and necropsied during pregnancy so that embryonic mortality, assumed to be due to chromosomal anomalies, may be characterized and quantified.

Development of Testing Strategies

Concern about adverse effects of mutation on human health, principally carcinogenesis and the induction of transmissible damage in germ cells, has provided the impetus to identify environmental mutagens. Genetic toxicology assays may be used to screen chemicals to detect mutagens and to obtain information on mutagenic mechanisms and dose-responses that contribute to an evaluation of hazards. Besides testing pure chemicals, environmental samples are tested because many mutagens exist in complex mixtures. The analysis of complex mixtures often requires a combination of mutagenicity assays and refined analytical methods.

The first indication that a chemical is a mutagen often lies in chemical structure. Potential electrophilic sites in a molecule are alerts to possible mutagenicity and carcinogenicity, because such sites confer reactivity with nucleophilic sites in DNA.

Assessment of a chemical's genotoxicity requires data from well-characterized genetic assays. Sensitivity refers to the proportion of carcinogens that are positive in the assay, whereas specificity is the proportion of noncarcinogens that are negative. Sensitivity and specificity both contribute to the predictive reliability of an assay.

Rather than trying to assemble batteries of complementary assays, it is prudent to emphasize mechanistic considerations in choosing assays. Such an approach makes a sensitive assay for gene mutations (e.g., the Ames assay) and an assay for clastogenic effects in mammals pivotal in the evaluation of genotoxicity. Beyond gene mutations, one should evaluate damage at the chromosomal level with a mammalian *in vitro* or *in vivo* cytogenetic assay. Other assays offer an extensive database on chemical mutagenesis (*Drosophila* SLRL), a unique genetic endpoint (i.e., aneuploidy; mitotic recombination), applicability to diverse organisms and tissues (i.e., DNA damage assays, such as the comet assay), or special importance in the assessment of genetic risk (i.e., germ cell assays).

Human Population Monitoring

For cancer risk assessment considerations, the human data utilized most frequently, in the absence of epidemiologic data, are those collected from genotoxicity/mutagenicity assessments in human populations. The studies conducted most frequently are for chromosome aberrations, micronuclei, and SCEs in peripheral lymphocytes.

The size of each study group should be sufficiently large to avoid any confounder having undue influence. Certain characteristics should be matched among exposed and unexposed groups. These include age, sex, smoking status, and general dietary features. Study groups of 20 or more individuals can be used as a reasonable substitute for exact matching because confounders will be less influential on chromosome alteration or mutation frequency in larger groups. In some instances, it might be informative to compare exposed groups with a historical control, as well as to a concurrent control.

Reciprocal translocations are transmissible from cell generation to generation, and frequency can be representative of an accumulation over time of exposure. The importance of this is that stable chromosome aberrations observed in peripheral lymphocytes exposed *in vivo*, but assessed following *in vitro* culture, are produced *in vivo* in hematopoietic stem cells or other precursor cells of the peripheral lymphocytes pool.

New Approaches for Genetic Toxicology

The ability to manipulate and characterize DNA, RNA, and proteins has been at the root of the advance in our understanding of basic cellular processes and how they can be perturbed. However, the development of sophisticated molecular biology does not in itself imply a corresponding advance in the utility of genetic toxicology and its application to risk assessment. Knowing the types of studies to conduct and knowing how to interpret the data remain as fundamental as always. There is a need for genetic toxicology to avoid the temptation to use more and more sophisticated techniques to address the same questions and in the end make the same mistakes as have been made previously.

Advances in Cytogenetics

Conventional chromosome staining with DNA stains such as Giemsa or the process of chromosome banding requires considerable expenditure of time and a rather high level of expertise. Chromosome banding does allow for the assessment of transmissible aberrations such as reciprocal translocations and inversions with a fairly high degree of accuracy. Stable aberrations are transmissible from parent to daughter cell, and they represent effects of chronic exposures. The more readily analyzed but cell-lethal, nontransmissible aberrations such as dicentrics and deletions reflect only recent exposures and then only when analyzed at the first division after exposure.

Specific chromosomes, specific genes, and chromosome alterations can be detected readily since the development of FISH. In principle, the technique relies on amplification of DNA from particular genomic regions such as whole chromosomes or gene regions and the hybridization of these amplified DNAs to metaphase chromosome preparations or interphase nuclei. Regions of hybridization can be determined by the use of fluorescent antibodies that detect modified DNA bases incorporated during amplification or by incorporating fluorescent bases during amplification. The fluorescently labeled, hybridized regions are detected by fluorescence microscopy. Alterations in tumors can also be detected on a whole-genome basis. Comparative genomic hybridization (CGH) has allowed an accurate and sensitive assessment of chromosomal alterations present in tumors. CGH is adapted for automated screening approaches using biochips.

The types of data collected will affect our understanding of how tumors develop. Data on the dose–response characteristics for a specific chromosomal alteration as a proximate marker of cancer can enhance the cancer risk assessment process by describing effects of low exposures that are below those for which tumor incidence can be reliably assessed. Cytogenetic data can also improve extrapolation of data generated with laboratory animals to humans.

Molecular Analysis of Mutations and Gene Expression

With technological advances, the exact basis of a mutation at the level of the DNA sequence can be established. With hybridization of test DNAs to oligonucleotide arrays, specific genetic alterations or their cellular consequences can be determined rapidly and automatically. cDNA microarray technologies allow the measurement of changes in expression of hundreds or even thousands of genes at one time. The level of expression at the mRNA level is measured by amount of hybridization of isolated cDNAs to oligonucleotide fragments from known genes or expressed sequence tags (EST) on a specifically laid out grid. This technique holds great promise for establishing a cell's response to exposure to chemical or physical agents in the context of normal cellular patterns of gene expression.

Conclusion

Genetic toxicology demonstrated that ionizing radiations and chemicals could induce mutations and chromosome alterations in plant, insect, and mammalian cells. Various short-term assays for genetic toxicology identified many mutagens and address the relationship between mutagens and carcinogens. Failure of the assays to be completely predictive resulted in the identification of nongenotoxic carcinogens. Key cellular processes related to mutagenesis have been identified, including multiple pathways of DNA repair, cell-cycle controls, and the role of checkpoints in ensuring that the cell cycle does not proceed until the DNA and specific cellular structures are checked for fidelity. Recent developments in genetic toxicology have improved our understanding of basic cellular processes and alterations that can affect the integrity of the genetic material and its functions. The ability to detect and analyze mutations in mammalian germ cells continues to improve and contribute to a better appreciation for the long-term consequences of mutagenesis in human populations.

Bibliography

Choy WN: *Genetic Toxicology and Cancer Risk Assessment*. New York: Marcel Dekker, 2001.

Mendrick DL, Mattes WB: *Essential Concepts of Toxicogenomics*. Totowa, NJ: Humana, 2008.

Sahu SC: *Toxicogenomics: A Powerful Tool for Toxicity Assessment*. Oxford: Wiley-Blackwell, 2008.

Smizarov D, Blomme E: *Genomics in Drug Discovery and Development*. Hoboken, NJ: John Wiley, 2009.

