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METHODS FOR EVALUATING CHEMICAL GENOTOXICITY

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INTRODUCTION

In toxicology, the term genotoxicity usually refers to any type of damage to the genetic apparatus (1, 2), the genome, just as cytotoxicity designates injury to the cell. Toxic effects to the genetic material have attracted much study for several reasons. The genome of germ cells, the reproductive cells, determines all heritable characteristics of organisms. The differentiated genome of the somatic cells that constitute an organism provides the information for the synthesis of most cellular components, and hence the maintenance of cellular integrity. Investigation of injury to the genome has led to the definition of a specific type of toxicity, genotoxicity, and to the development of a sub-specialty, genetic toxicology (3, 4).

Genotoxic effects of chemicals can lead to a variety of disease states in experimental animals or humans, ranging from those brought about by cell death to those stemming from cell immortalization. These include acute toxicity, reproductive effects, birth defects, heritable diseases, cancer and possibly autoimmune conditions, and certain degenerative diseases. In the assessment of chemical safety, therefore, it has become standard to evaluate genotoxicity, usually by several means. The requirements of governmental regulatory agencies vary considerably (5), depending to a large extent upon the hazards inferred from genotoxicity.

The methods for measuring genotoxicity are numerous and varied (6, 7). This review describes the principal types of genotoxicity, involving effects on DNA, genes and chromosomes, and some of the more commonly used systems to measure specific types of genotoxicity (Table 1). The review does not address the strategy for deployment of short-term tests because this is a separate topic that must take into account a number of factors, including the

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objectives of testing, for example, detection of germ cell mutagens (2) or carcinogens (8); practical considerations, such as the economic and ethical consequences of using whole animals for screening; and complimentarity of endpoints and biotransformation capability of different tests that might be considered for a battery.

DNA DAMAGE

Chemicals can damage DNA in several ways, including base damage, cross-linkage, and strand breakage, as a result of reaction with constituents of DNA or other types of interaction, such as intercalation. DNA contains an abundance of nucleophilic atoms, mainly carbon, nitrogen and oxygen, and consequently is readily attacked by electrophiles. Several types of chemicals are electrophiles, notably industrial intermediates, and many more can be converted to electrophiles by enzymatic biotransformation in animals. It has long been possible to deduce from the structure of a chemical the likelihood of its acting as an electrophile (9–11) and this has made for quite accurate predictions of the capacity of chemicals to react with DNA. Chemicals can also damage DNA by causing the formation of reactive oxygen species (12, 13).

Damage to DNA can be measured directly by a variety of biochemical or biophysical techniques. Some of these, such as the binding of radiolabeled

Table 1 Availability of assays for genotoxicity

	Germ Cell			Somatic Cell		
	DNA damage	Gene mutation	Chromosome alteration	DNA damage	Gene mutation	Chromosome alteration
Prokaryote	NA	NA	NA	+	+	NA
Submammalian Eukaryote						
in vitro	–	–	–	+	+	+
in vivo	+	+	+	+	+	+
Mammalian						
in vitro	–	–	–	+	+	+
in vivo	+	+	+	+	+	+
Human						
in vitro	–	–	–	+	+	+
in vivo	m	m	m	m	m	m

NA = Not applicable
\+ = available
– = not available
m = Monitoring approach

compounds or radioimmunoassay using antibodies to modified DNA (14), are limited to measurement of specific adducts. Others, such as alkaline (15, 16) or neutral (17) sucrose gradients, alkaline unwinding (18, 19), or alkaline elution (20), which measure breakage, and fluorimetric techniques (21), high performance liquid chromatography (22, 23), mass spectral analysis (24), and ^{32}P-postlabeling (25), which measure modified bases, can identify DNA damage of unknown types. Recently, a rapid DNA precipitation technique has been reported for measuring strand breaks (26).

Reaction of a chemical with DNA can lead to breakage of the DNA upon isolation as a consequence of fragility of the DNA or depurination or, less commonly, depyrimidation. Breakage can be determined by measurement of the size of single strand DNA by alkaline sucrose gradient centrifugation, in which small molecular weight fragments are separated from large molecular weight DNA (16), or by alkaline elution from filters that retard passage of large molecular weight DNA while allowing small molecular weight DNA to pass through (20). The size of double strand DNA can be measured by neutral sucrose gradient centrifugation (17). In principle, these techniques should be very sensitive measures of DNA damage since a single break in the DNA strand gives rise to reduced molecular weight fragments. However, reduction in the size of DNA due to strand incision can be difficult to distinguish from nucleolytic degradation or mechanical fragmentation of DNA. Accordingly, caution must be used in the interpretation of fragmentation. Nevertheless these approaches have proven useful for chemical screening (27–29).

A technique coming into ever greater use because of its versatility is ^{32}P-postlabeling. This method, developed by Randerath and coworkers (25), involves isolation of DNA followed by enzymatic degradation to nucleosides. Unaltered nucleosides are removed by extraction and the remaining nucleosides containing adducts are labeled at the 5'-end with ^{32}P-ATP in a reaction catalyzed by T4-kinase. Labeled adducts are separated by multidirectional polyethyleneimine cellulose thin layer chromatography and visualized as spots on film autoradiography (30).

Measurement of repair of DNA damage or the consequences to DNA function of damage, such as inhibition of DNA replication or selective killing of repair-deficient cells, can also be used to indirectly assess DNA damage.

The types of DNA repair, i.e. excision, O^6-alkylguanine-DNA-alkyltransfer, postreplication, and photoreactivation, and the variety of ways in which these can be measured have been the subject of numerous detailed reviews (31–33). The major types of DNA damage all elicit excision repair and, at present, only this type of repair has significant application to monitoring DNA damage.

Different types of excision repair were described by Regan & Setlow (34) as long patch and short patch repair. Presently, two main types of excision

repair are now recognized: nucleotide excision and base excision. The O^6-alkylguanine-DNA-alkyltransferase type of excision repair is a very specific process involving transfer of the alkyl group from guanine alkylated at the O^6 position to an acceptor site on the protein (35), and hence is not useful for genotoxin detection.

In nucleotide and base excision, the initial step differs, but both result in incision of the DNA strand in the vicinity of the damage, excision of a stretch of DNA containing the region of damage, synthesis of a patch, and rejoining of the strand. Each of these processes can be used as a measure of the DNA damage produced by a chemical.

The first step in base excision is the spontaneous or N-glycosylase-mediated removal of the damaged base. This is followed by the action of an apurinic-apyrimidinic endonuclease. In nucleotide excision repair, the first step is incision by an endonuclease in the vicinity of the DNA damage. Both processes lead to discontinuities in the DNA chain which can be measured as a reduction in the size of either single or double strand DNA by the techniques discussed for measurement of DNA damage.

The next process in repair is excision of a portion of DNA by an exonuclease. The amount of removal varies depending upon the type of damage and the type of excision repair that is provoked (31). Specific determination of excision requires measurement of a loss of bases from DNA (36) or a reduction in the amount of chemical adducts in DNA (37–39). The latter is more frequently done, but requires either that the genotoxin be labeled with a radioisotope or that some other means of measuring the amount of adduct be used. Where such measurements are possible, they are usually employed to determine adduct formation, as discussed above, rather than removal. An alternate approach, more applicable to monitoring adduct removal, is the measurement of the loss of sites sensitive to enzymes that incise DNA in the region of damage (40). Here, damaged DNA is incubated with purified endonucleases, and the number of damaged sites is measured by the action of these enzymes in reducing the size of DNA, usually revealed by alkaline sucrose gradient centrifugation. DNA damage by several types of chemical has been detected in this manner (41–43). The major limitations are the requirement for the appropriate nuclease and the minimal information available on the susceptibility of different types of chemical damage to incision by nucleases in vitro.

Following the excision of damaged segments of DNA, patches are synthesized by using the opposing strand as template. This process can be measured by incorporation of nucleotides into the newly synthesized DNA by a variety of techniques. Originally, repair synthesis in mammalian cells was demonstrated by autoradiographic incorporation of ^{3}H-thymidine as unscheduled DNA synthesis (UDS), that is, DNA synthesis that is not confined to the DNA replicative phase of the cell cycle (44). This procedure still remains the

most frequently used for assessing repair synthesis provoked by chemicals. Autoradiographic measurement of repair offers the advantage that only a light labeling over the nucleus due to ^{3}H-thymidine incorporation during repair is produced and this is easily distinguished from the heavy nuclear labeling of the quantitatively greater replicative DNA synthesis. Autoradiographic UDS has been shown to correspond to repair synthesis in the nonreplicating strands of parental DNA (45, 46).

UDS can also be measured by liquid scintillation counting of radioactive thymidine incorporation into the DNA of cells in which replicative DNA synthesis has been reduced by hydroxyurea (45), arginine deficiency (47), or a combination of both (48). The hydroxyurea block has been extensively used (44, 47, 48). Some difficulties, however, may arise with this technique. Replicative DNA synthesis is not completely arrested by hydroxyurea (46), hence there is a lack of certainty on the significance of increases above the persisting background. The sensitivity of this approach may be limited, since DNA damage will further inhibit the replicative synthesis persisting in the presence of the block, and, thus, repair will not become evident until the repair synthesis results in thymidine incorporation sufficient to equal the inhibited replicative synthesis and thereby exceed the control level. Another complication is that other chemicals can interfere with hydroxyurea suppression of replicative DNA synthesis (49) and restore replicative synthesis that can be confused with repair. Finally, under some conditions, hydroxyurea has induced DNA repair (50, 51). It has also been used to inhibit DNA synthesis for autoradiographic measurement of UDS (52), but suffers from the same uncertainty on the determination.

Several techniques have been developed which definitively distinguish repair synthesis from replicative synthesis. Incorporation of the thymidine analog, 5-bromo-2'-deoxyuridine (BrdUrd), into regions of repair is not sufficient to alter the density of parental DNA, whereas replicated DNA is rendered denser due to more extensive incorporation. This difference in density between parental and replicated DNA allows them to be separated on cesium chloride density gradients. Additional incorporation of either radioactive thymidine or BrdUrd into the isolated parental DNA provides a measure of repair synthesis (53–55). BrdUrd incorporation can also be measured by using immunochemical staining of antibodies to BrdUrd (56).

Other techniques for estimating the extent of repair involve the photolysis of 5-BrdUrd incorporated during repair (57) and the measurement of thymidine incorporation into DNA growing points that are retained on benzoylated naphthoylated DEAE-cellulose columns because of their single stranded regions (58). These techniques have not found wide usage in screening, probably because of their rather demanding technical requirements. Nevertheless, they provide a reliable measurement of chemically-induced DNA repair.

The final process in excision repair is rejoining of the strand by a ligase.

This process can be followed by observing the restoration of fragmented DNA to full length by using gradient centrifugation (59).

Evaluation of the various approaches to the measurement of DNA repair has led to the conclusion that autoradiographic measurement of UDS is the most appropriate technique for screening (60). The EPA Gene-Tox review of the usefulness of DNA repair assays other than UDS also concluded that these techniques were less suitable for screening (61). Autoradiographic measurement of UDS is simple, reliable, and also affords a quantification of the fraction of cells responding.

In Vitro Tests: DNA Damage

PROKARYOTIC Various systems have been used to measure DNA damage. A useful test in bacteria is the E. Coli pol A^- system developed by Rosenkranz (62). This test uses a pair of isogenic strains differing in that one strain, pol A^-, is deficient in DNA polymerase I, which is required for DNA repair. Thus, the deficient bacteria are preferentially killed by DNA-damaging compounds.

A similar test system in Bacillus subtilis uses strains competent or deficient in the rec A gene product that facilitates cleavage of the lex A repressor of genes of the SOS system. Agents showing greater lethality to the Rec^- strain are presumed to have caused DNA damage (63). The recently developed SOS chromotest (64) is a system in which DNA damage in E. Coli elicits induction of the sfi A gene as part of the SOS system and increases expression of β-galactosidase, which is measured colorimetrically.

Bacteria have little biotransformation capability, except for aryl nitro reduction (65). Thus, these assays, as well the Ames test for which enzyme supplementation was first routine, use mammalian enzyme preparations, usually a liver subcellular preparation, for bioactivation of most chemicals.

EUKARYOTIC Cultured mammalian cells are commonly used to assess the DNA damaging activity of chemicals. Direct measurement of DNA damage can be made by any of the techniques described. Alkaline sucrose gradient techniques and alkaline elution have been commonly used (26–28), but ^{32}P-postlabeling is coming into greater use (66). Various cell types have been used (67), but all, except liver cell lines (68) or hepatocytes (28), require an exogenous system for bioactivation.

One aspect of screening for DNA damage is that repair occurs quite rapidly in some systems and this necessitates the study of multiple time points in order to provide convincing evidence of lack of genotoxicity. An approach to preserving DNA lesions would be the use of inhibitors of repair to allow damage to accumulate (69).

DNA damage impedes the activity of DNA polymerases and consequently

inhibits replicative DNA synthesis, which, in turn, has been used as an index of DNA damage. R. B. Painter (70) developed an approach using HeLa cells in which DNA damage was distinguished from other types of inhibition of DNA synthesis by the rapid rate of recovery of thymidine incorporation after the removal of an inhibitor, in contrast to a slow rate of recovery after exposure to a genotoxin. Similar approaches can be used in organ cultures (71).

DNA Repair

Excision DNA repair by mammalian cells was demonstrated in 1966 by Rasmussen & Painter (72) in cultured cells. Subsequently, it has also been measured in organ fragments (73), but cultured cells have been the system most widely used for screening tests (67). Among those currently used for chemical studies, freshly isolated hepatocytes, as originally described by G. M. Williams (74, 75), have become perhaps the most widely used (76) because of their high capacity to activate genotoxins. Hepatocytes from various species (75), including humans (77, 78), have been used.

Although bacteria have proven valuable in the study of the enzymology of DNA repair, they have not been extensively used for the testing of chemicals by measurement of DNA repair, probably because they are commonly used for other approaches and mammalian cells have proven so useful for DNA repair assays.

In Vivo Tests: DNA Damage

All the techniques described for measurement of DNA damage in vitro can be applied in vivo. The advantages here are that biotransformation of the whole organism is taken into account and that various organs can be evaluated. On the other hand a number of animals must be used to obtain data at several concentrations and time points.

Systems are available for the detection of effects on DNA in germ cells, and somatic cells. Among germ cell assays, measurement of DNA fragmentation using alkaline and neutral filter elution has been applied (79). Inhibition of testicular cell DNA synthesis has also been used (80, 81). For the measurement of somatic cell DNA damage, a facile approach is the measurement of DNA fragmentation in cells from liver and other tissues by alkaline sucrose gradients (82) or alkaline elution (83, 84).

DNA Repair

Measurement of UDS has been applied to assessment of chemical genotoxicity in vivo. Tissues studied include stomach (85), skin (86), kidney (87), and liver (88). The usual approach is to expose the animal to the test chemical and then isolate tissue fragments or cells and provide them with ^{3}H-thymidine for

incorporation during repair synthesis. Several approaches have been developed for the measurement of chemically-induced DNA repair in spermatocytes (89, 90) or oocytes (91).

Human Monitoring

Exposure of humans to DNA-damaging chemicals can be detected in a variety of ways. A limitation of all measurements is that damage is maximal at the time of exposure and then diminishes over time as repair proceeds. Antibodies are available for detecting the DNA adducts of about 15 chemicals (92). Genotoxins also form adducts with proteins and one approach to monitoring exposure is the measurement of hemoglobin adducts (93–95). Since there is no repair of hemoglobin adducts, they persist longer than DNA adducts. The ^{32}P-postlabeling technique offers the advantage that neither the chemical nor the adduct needs to be known (96).

GENE MUTATION

A mutation is a transmissable change in genetic material involving alteration in the structure, arrangement, or number of genes. The change can be discrete, involving a single base pair, as with point mutations, or rather gross, such as a chromosomal alteration. This section deals with structural mutations in genes. Other types of mutations are covered under chromosomal alterations.

Mutations in germ cells are heritable mutations that can be transmitted to subsequent generations, whereas mutations in somatic cells affect only the exposed generation. All germ cell mutagens are also somatic cell mutagens, but the converse is not true. The principal reason appears to be that germ cell mutations can be measured only in vivo and hence are more difficult to achieve.

Mutants are identified by some change in phenotype. In vitro, mutants are usually selected by their ability to survive in the presence of a toxin that kills wild-type cells, or by their ability to grow under conditions that are inadequate for the parental genotype.

For the most part, the types of chemicals that produce DNA damage also produce mutations as a consequence of the DNA damage. However, other types of chemicals have also been reported to be mutagenic.

In Vitro Tests: Prokaryotic

Of a variety of bacterial mutagenicity tests that have been developed (97, 98), by far the most commonly recommended and used is the Ames test, developed by B. N. Ames, which measures back mutation to histidine independence of histidine mutants of Salmonella typhimurium. A number of

tester strains are available, including some with abnormalities in the cell wall to make them permeable to chemicals, some deficient in DNA repair, or some that carry an R factor-enhancing mutagenesis (99). Strains sensitive to oxidative mutagenesis are available (100). As mentioned above, bacteria require enzyme supplementation for bioactivation of xenochemicals.

The test can be performed by the standard plate assay or by a sensitive variant involving turbidity measurement, the fluctuation test (101). Its capability of detecting certain genotoxins has been enhanced by preincubating the chemical and the activation system with the test organism (102). Shortcomings of the standard Ames test are that it does not quantitate mutational frequencies, unlike mammalian mutagenesis assays, and cytotoxicity is not easily quantified. Efforts have been made to counter these deficiencies (103), but, nevertheless, a small increase in recovery of revertants under test conditions that are highly toxic to the organisms must be interpreted cautiously.

The Ames test measures back mutations to histidine independence in specific mutated sequences that confer histidine dependence. Since multiple modes of reversion are possible for each strain, inferences about the nature of molecular changes must be cautious. Nevertheless, the target sequences for mutagenesis are known and within these there are highly mutagen-susceptible nucleotide stretches (104).

In Vitro Tests: Eukaryotic

Mutations can be measured in several types of fungi, including the yeasts, Saccharomyces cerevisiae, Schizosaccharomyces pombe and the molds, Neurospora crassa and Aspergillus nidulans (105). Fungi possess some intrinsic chemical biotransformation capability and various types of genetic effects can be delineated.

Gene mutations at a number of loci can be measured in mammalian cells (106, 107). The three assays most widely used for screening are mutagenesis at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus (106), thymidine kinase (TK) locus (108), or plasma membrane Na/K-adenosine triphosphatase (ATPase) locus (109, 110). Mutants are identified by their resistance to toxic chemicals that are substrates for the enzymes coded for by target loci and that thereby kill enzyme-competent cells.

HGPRT mutagenesis is a widely used endpoint (111, 112). In this assay, mutants lacking the purine salvage pathway enzyme are identified by their resistance to toxic purine analogs, such as 8-azaguanine or 6-thioguanine. This assay has the advantage over ATPase mutagenesis, measured as ouabain resistance, in that it involves a nonessential function, unlike the membrane ATPase system, and consequently there are no lethal mutants, which would be undetected. Its advantage over the measurement of TK-deficient mutants by resistance to thymidine analogs is that the gene for HGPRT is on the

X-chromosome rather than a somatic chromosome, as with thymidine kinase, and hence only one functional copy is present in each cell. Because of this feature, HGPRT-deficient mutations in wild-type cells can be determined, whereas a heterozygous mutant is required for measurable mutation to homozygous TK deficiency. A possible drawback of HGPRT mutagenesis is that deletion mutagens might not be detected since the cells would be killed. Both gene and chromosomal mutants have been identified in the TK locus (108), although the utility of this feature for genotoxin detection has not yet been established.

Mutagenesis at any of the three loci involves a forward mutation, unlike mutagenesis in the Ames test, and the genomic target also is larger and less specific. Moreover, molecular analysis of the nature of mutations in the HGPRT and TK genes can be performed (113).

The target cells used in purine analog resistance assays have mostly been fibroblast-type, such as the V79 and CHO lines (111, 112). These lines possess little ability to activate genotoxins other than polycyclic aromatic hydrocarbons. This deficiency has been overcome by providing exogenous metabolism mediated by either enzyme preparations (114, 115) or cocultivated cells (115, 116), including hepatocytes (117, 118). HGPRT mutagenesis can also be measured in liver epithelial cells, which have biotransformation capability (119), or in human cells, which can be combined with hepatocytes for biotransformation (120). The latter system provides clear evidence of effects on the human genome, although thus far mutability of human cells has not differed qualitatively from that in other animal cells.

In Vivo Tests

Systems are available for identifying mutations in either germ cells or somatic cells.

Fruit flies, Drosophila melanogaster, have been used in mutagenicity studies for over 50 years. Drosophila are sexually reproducing organisms with male and female germ cell stages equivalent to those of mammals. As a result of extensive studies, the genetics of Drosophila are better understood than those of any other higher eukaryote. Several assays have been developed (121), of which the most widely applied is the sex-linked recessive-lethal test for germ cell mutations, originally devised by H. J. Muller. This test is usually performed by exposing wild-type males and mating them to a tester strain of females that carry genetic markers on the X chromosome affecting the shape and color of the eyes. The F_1 females carry both the exposed and tester strain X chromosomes. These females are mated with F_1 males and the F_2 generation is scored for the absence of males, indicating recessive lethality.

Mammalian germ cell mutation assays include the mouse-specific locus

test, measurement of mutant proteins by electrophoresis, the dominant lethal test, and the heritable translocation test. The last two are tests for chromosomal mutations and are described in a subsequent section. The specific-locus test developed by W. L. Russell (122) basically involves exposing a male mouse that is homozygous for the dominant gene for dark coat color at a specific locus and then mating it with a female that is homozygous for the dilute coat color recessive gene at the same locus. Mutation in one of the dominant genes will yield dilute colored offspring. A limitation is that the number of loci in one strain that are available for mutation analysis is small, i.e. seven in Russell's strain, and hence a large number of mice are required, making it impractical for routine screening. Nevertheless, the specific-locus test is the definitive test for germ cell mutations (123).

Gel electrophoresis of enzymes and proteins has been used to detect qualitative and quantitative changes indicative of mutations (124–127). Liver proteins are usually studied because many more proteins can be visualized than with other tissues. Typically, male mice are exposed and then mated with nonexposed females of a different strain, and liver samples are taken from the offspring. Crossing is done to maximize the number of heterozygous loci, since mutations will most likely occur at these and X-linked loci where only one allele would need to be affected. This approach has recently been improved by computer assisted analysis of the electrophoretic patterns (127). Although this is a powerful tool, it is limited to detection of mutations that lead to a change in the charge of the affected protein.

Several in vivo somatic cell mutation assays are established. HGPRT mutants have been isolated from several tissues of hamsters exposed in vivo (128). Based on earlier studies of mutants in human peripheral blood lymphocytes (see below), a clonogenic assay for HGPRT mutant mouse lymphocytes was developed (129).

The mouse spot test (130) detects mutations in genes controlling hair pigmentation. It consists of exposure of mouse embryos in utero that are heterozygous for recessive coat-color alleles. Mutation in a wild-type allele at one of the heterozygous coat-color loci in melanocyte precursors results in colored patches of hair observable in newborns at two weeks of age. This test thus assesses the capacity of a mutagen to penetrate the placental barrier. However, relatively few chemicals have been examined in this test (131).

Human Monitoring

Of the various genotoxicity assays, mutagenesis probably has the greatest potential for monitoring exposure to genotoxic chemicals. This is because mutations are persistent, unlike DNA damage or chromosome aberrations, and mutated cells can undergo clonal expansion. At present, the approach introduced by R. J. Albertini is receiving considerable attention: in this,

HGPRT mutant lymphocytes in peripheral blood are detected by measuring 6-thioguanine resistance in vitro. (132, 133). Enzyme variants in erythrocytes have been described by J. V. Neel and coworkers (134), but this has not been applied to detection of mutations in individuals. In principle, mutations in the cells of organs that produce serum proteins could be identified by the detection of abnormal proteins using two dimensional electrophoresis, as has been done in experimental animals.

Various body products and fluids, especially urine, can be assayed for the presence of mutagens in the Ames test or other systems (135, 136).

CHROMOSOME ALTERATIONS

Alterations of chromosomes can be both structural (aberrations) and numerical (aneuploidy). Both types represent mutations, but unlike gene mutations do not involve structural changes in genes. The underlying mechanisms of chromosomal mutations are beginning to be elucidated in some detail (137, 138), although not to the same extent as for DNA damage or gene mutation. It is now known that, in general, DNA-reactive chemicals produce aberrations, whereas chemicals that disturb the mitotic spindle produce aneuploidy. The latter type of genotoxicity can be detected only by cytogenetic assays. Clearly, cytogenetic assays reveal effects not observed in other systems; for example, a substantial number of chemicals are clastogenic to mammalian cells, but not mutagenic to bacteria (139).

Determination of effects on chromosomes is of conceptual importance because chromosome alterations are indicative of damage at a higher level of genetic organization than DNA damage or gene mutations. The effect of a specific chemical on chromosomes cannot be deduced from structure as reliably as can DNA reactivity and mutagenicity.

Definitive chromosome tests require laborious karyotype analysis of metaphase spreads, as well as the expertise of a cytogeneticist to identify specific alterations in chromosome structure. Nevertheless, a unique feature of cytogenetic analysis is that special staining techniques for identifying the organization of individual chromosomes permit analysis of effects at specific sites within chromosomes (138). This is of particular importance in respect to the proximity of oncogene sequences to fragile sites (140). However, for simplicity in general screening, several alternatives have been developed, of which the measurement of sister-chromatid exchanges (SCEs) is perhaps the most commonly used. SCEs are regarded as interchanges between DNA molecules at homologous loci within a replicating chromosome, although the molecular events at the points of DNA interchange have not been delineated. Exchanges are readily identified in sister chromatids differentially stained by

BrdUrd-dye techniques. Measurement of SCEs has shown sensitivity to genotoxins not readily detected by clastogenicity (141).

A potential limitation of cytogenetic analysis of metaphase spreads is that the effect leading to aberrations must be present at the time of metaphase. If the effect is due to DNA damage, repair may occur before the cell reaches metaphase. An alternative is the measurement of premature chromosome condensation in interphase (142), which can be induced in interphase cells by fusion with inducer cells.

In Vitro

Only in eukaryotic cells is the genome organized into chromosomes and thus chromosomal tests are done only with cells at this phylogenetic level. Most in vitro test systems use mammalian cells, often human, because effects on this cell type are of most interest to potential human hazards. In vitro systems are thus far limited to study of effects on somatic cells.

Mammalian cell cytogenetic studies are usually done with blood leukocytes stimulated to divide by mitogens or with sublines of Chinese hamster ovary (CHO) cells (143). The latter are favored because of their low chromosome number, i.e. 20–22 as compared to 40 or greater in the mouse, rat, and human. For in vitro studies with lymphocytes, human cells are preferred over rodent. The assessment of chemically induced SCEs is usually made in CHO or V79 cells (141), again because of the simplicity of their karyotype.

Established cells lines are not appropriate for study of induction of aneuploidy because most lines become aneuploid. Normal Syrian hamster embryo primary cultures, which are diploid, have been used successfully (144). Another recently developed approach is the use of monochromosomal hybrid cell lines (145). The principle of this technique involves the development of human/mouse hybrid cell lines containing a single human chromosome with a gene coding for xanthine-guanine phosphoribosyl transferase that permits selection of cells with the chromosome and those that have lost it. The cloning efficiency of cells in a medium containing 6-thioguanine, which kills cells with xanthine-guanine phosphoribosyl transferase activity, provides a measure of loss of the chromosome, i.e. aneuploidy.

All these cell types require supplementation with an exogenous system for biotransformation of most xenochemicals. A liver subcellular preparation is most commonly used (143). Whole cell systems can also be used to mediate SCE induction in cocultured target cells, including human cells (146, 147). In addition, liver cell systems with intrinsic activation capability have been described (148–150).

In the interpretation of SCEs or other types of chromosome damage, a large number of culture factors can influence the frequency of damage (137, 151–153). Moreover, with SCEs it has been established that the background

is a function of the extent of incorporation into DNA of the BrdUrd for the differentiation of sister chromatids (141) and, thus, changes in incorporation can alter the level of exchanges measured (154). Consequently, low level responses, especially at high levels of BrdUrd incorporation, must be interpreted with caution.

Another relatively simple test for chromosomal alterations is the micronucleus test. It can be performed on a variety of cultured cell types (155), but is usually done as an in vivo test.

In Vivo

There is a generally good correlation between in vitro and in vivo cytogenetic assay results, although more chemicals are positive in vitro than in vivo (156). Some of the positive in vitro results may be due to the artifactual conditions resulting from exposures to high concentrations (153). Thus, in vivo assays serve the important purpose of clarifying "false positives."

In vivo tests can assay effects on germ cells or somatic cells. Chromosome alterations can be assessed in male and female germ cells by cytogenetic techniques (143). Two assays for germ cell chromosomal mutations are the dominant lethal test and the heritable translocation test. In the former (157), male mice are typically exposed to the test chemical and then mated. Early fetal deaths are considered the most reliable parameter of a dominant mutation that is lethal to the fetus that is heterozygous for it. Chromosome breakage is accepted as the primary genetic lesion responsible for dominant lethals.

In contrast to measuring lethal mutations, a specific type of chromosomal alteration, translocations, is measured in viable progeny (158). The basis for the heritable translocation test is that less than one third of all male translocation heterozygotes are sterile and the remainder partially sterile. Thus, male mice are exposed throughout spermatogenesis and then mated. The male progeny are scored for complete or partial sterility, the latter determined by dead placental implants in impregnated females.

For in vivo somatic cell cytogenetic assays, the cell type most commonly used is rodent bone marrow (143). Peripheral blood leukocytes are also commonly used.

Micronuclei are small stainable bodies outside the nucleus, a result of the exclusion from the nucleus of a partial or whole chromosome. By measurement of micronuclei, chromosome damage to cells in bone marrow, spleen, or peripheral blood of mice can be assayed (159, 160).

SCEs have been studied in a variety of nonmammalian systems (141). For mammalian studies, mice are preferred, probably because their small size requires minimal BrdUrd for differentiation of sister chromatids. In the mouse, bone marrow cells are usually studied.

Human Monitoring

The most widely applied technique for studying exposure to genotoxic chemicals is probably the measurement of chromosome aberrations (161). Detection of SCEs has also been successfully used (162). Detection of micronuclei (163) offers a particular advantage inasmuch as micronuclei can be persistent and consequently are found in nondividing progeny of proliferating cells.

INTERPRETATION OF GENOTOXICITY

Genotoxicity, broadly defined as damage to the genome, is a distinct and important type of toxicity. A wide variety of systems are available for studying DNA damage, gene mutations, and chromosomal alterations in germinal and somatic cells, both in vivo and in vitro (Table 1). Many of the in vitro tests for genotoxicity have close in vivo counterparts, which allow specific comparisons of results in the two systems. The correlation is generally good, but discrepancies are evident: these arise mainly where in vitro systems fail to mimic in vivo biotransformation or where in vitro systems suffer from an antifact, such as alteration of the culture milieu by high concentrations of chemicals. The recognition of such complexities should guide interpretation of observations.

The early work in the field was concerned with understanding and predicting human germ cell mutations. A major finding is that no human genetic disease can yet be traced to genotoxic chemicals. One reason may be that germ cells are less accessible for chemical attack than are somatic cells. Consequently, most environmental exposures appear to be at such levels that would require the study of very large populations in order to detect mutations.

Perhaps the most common use of genotoxicity tests is to predict carcinogenicity (60, 67, 164, 165). Early enthusiasts were persuaded that carcinogens are mutagens and this idea achieved the status of a paradigm, which persists at the present. Many carcinogens are indeed mutagens, as has been thoroughly demonstrated in the systems reviewed here. In most instances this is attributable to the ability of such chemicals to act as electrophiles or free radicals. Nevertheless, there are experimental carcinogens without such properties that instead act through other processes, including promotion of the neoplastic process. These have been referred to as epigenetic carcinogens to distinguish them from the DNA-reactive or genotoxic type (10). This distinction is of considerable importance in interpreting genotoxicity results. DNA-reactive carcinogens are generally readily detected in a battery of short-term tests encompassing different endpoints and biotransformation systems (166). Epigenetic carcinogens are by definition negative in tests for DNA-reactivity, although they may yield positive results for certain genotoxic effects, such as chromosomal alterations, because of their

perturbation of cellular processes. Negative results in short-term tests thus do not constitute evidence of noncarcinogenicity in animals, although the likelihood is greater than if the chemical were genotoxic. In fact, there is no adequate documentation of a chemical that forms adducts in DNA but is not carcinogenic in experimental animals.

Several hundred chemicals have been documented to be carcinogenic in animal models (167). Of these, only about 50 have been associated with cancer in humans. Most of the human carcinogens are DNA-reactive agents, such as industrial chemicals and alkylating chemotherapeutic drugs (168). Thus, genotoxicity tests serve the important function of identifying carcinogens with the greatest potential human hazard. Such a judgment, however, needs to take into account other characteristics of a carcinogen (169). Moreover, with the availability of realistic human systems in which to study genotoxic effects, it has been proposed that experimental carcinogens that are genotoxic in such systems should be regarded as presumptive human carcinogens (166).

Although chemicals can clearly cause cancer in humans, the contribution of industrial chemicals to the cancer burden is much lower than the attention given to the problem (170) would suggest. To the contrary, it is becoming increasingly clear that voluntary lifestyle factors are major elements in the multifactorial etiology of the main human cancers (171, 172). In the future, it can be anticipated that many of the approaches reviewed here, especially human monitoring techniques, will be applied to the study of the involvement of lifestyle in cancer causation.

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