

## Current Trend of Advanced Methods in Cellular and Molecular Toxicology

Jae-Chun Ryu

*Toxicology Laboratory, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul 130-650, Korea*

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### Introduction

The detection and the regulation of man-made synthetic chemicals are subjects of great concern in administrative authorities because of its close correlation between environmental contamination and human health. It has been widely assumed that mutation represents at least one step in carcinogenesis. The evidence supporting this idea is that the majority of mutagens are carcinogens (McCann *et al.*, 1975) and, for some compounds, mutagenic potency is closely correlated with carcinogenic potency (Meselson and Russel, 1977). The evaluation of toxicity that may pose a genetic hazard in our environment becomes the center of concern lately. Since the tens of thousands of man-made chemicals have been introduced into the environment in the last few decades, it is necessary to examine whether the chemicals cause the effect of DNA damage. Generally, the carcinogenicity including genotoxicity is one of the potential toxicity that may be considered for the human health. Moreover, mutagens and certain non-mutagenic carcinogens have also been found to induce chromosomal rearrangement (Zimmermann, 1971), which may affect carcinogenesis by altering gene expression, perhaps by allowing the activation or inactivation of cellular cancer genes (Radman *et al.*, 1982). Several assay systems with rapidity and reliability have been introduced for this purpose, such as reversion test with bacterial gene mutation (Ames *et al.*, 1973, 1975; Maron and Ames, 1983), chromosomal aberration assay with mammalian cells (Ishidate and Odashima, 1977), and micronucleus assay with rodents (Hayashi *et al.*, 1992; Schmid, 1975). These assay systems are now well used to evaluate the

genotoxicity of chemicals, and also frequently adopted as methods for an index of genotoxicity worldwide. Furthermore, it is well applied as a screening probe for the detection of possible carcinogenic substances in our environment.

Practically, several short term methods have been applied (Ames *et al.*, 1973; Maron and Ames, 1983; Mersch-Sundermann *et al.*, 1991) for predicting the carcinogenicity of chemicals and also been introduced to the evaluation of genotoxicity (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979; Radman *et al.*, 1982; Hayashi *et al.*, 1982, 1990, 1992; Ryu *et al.*, 1993a, 1994a,b, 1996a,b, 1998b,d, 2001b; Kim *et al.*, 2001) and of antimutagenicity (Sato *et al.*, 1991; Ryu *et al.*, 1993b, 2001c). Cytogenetic studies on mammalian cells *in vitro* (Schmid, 1975; Hayashi *et al.*, 1982, 1990, 1992, 1994a; Heo *et al.*, 1997) as well as *in vivo* (Ishidate and Odashima, 1977; Matsuoka *et al.*, 1979) have also been widely used as a screening method for DNA-attacking substances.

However, these kinds of toxicity evaluation tools cannot elucidate the mode and/or mechanism of actions of chemicals, especially carcinogens and mutagens. Recently developed transgenic mutagenesis assay system coincides with subjected for this purpose because this assay system may provide a powerful tool to predict the mutation spectrum induced in cancer-related genes more accurately. Moreover, many scientists try to develop more precise, convenient and sensitive techniques for the detection of DNA damages as an index of carcinogenicity.

Recently, several new methods for the detection of genetic damages *in vitro* and *in vivo* were introduced according to the rapid progress in toxicology combined with cellular and molecular biology. Among these methods, the single cell gel electrophoresis (comet) assay which

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\*To whom all correspondence should be addressed

can detect DNA damages in cell level (Mckelvey-Martin *et al.*, 1993; Singh *et al.*, 1994; Ryu *et al.*, 1997, 2001e; Tice *et al.*, 2000; Lee *et al.*, 2001), mouse lymphoma thymidine kinase gene assay (Clive *et al.*, 1983; Sawyer *et al.*, 1985; Ryu *et al.*, 1999a), FISH (fluorescence *in situ* hybridization) (Hayashi *et al.*, 1994b), PRINS (primed *in situ* hybridization) (Abbo *et al.*, 1993) and transgenic animal and cell line model as a parameter of *lac I* (Big Blue) (Kohler *et al.*, 1991; Ryu *et al.*, 1998a,c, 1999a, 2000, 2001d, 2001f) or *lac Z* (Muta Mouse) (Suzuki *et al.*, 1993) gene mutation are newly introduced based on cellular and molecular toxicological approaches. Also, *in vivo* supravital micronucleus assay with peripheral reticulocytes by using acridine orange fluorescent staining (Hayashi *et al.*, 1990, 1992; Ryu *et al.*, 1998b) was introduced instead of mouse bone marrow micronucleus assay.

Especially, International Workshop on Genotoxicity Test Procedure (IWGTP) was held March 25-26, 1999 in Washington DC, as a satellite meeting of the 30th Annual Meeting of Environmental Mutagen Society supported by OECD and ICH. The objectives of the workshop were threefold: (1) to establish uniformity regarding the conduct of specific assays; (2) to make progress toward standardizing procedures where possible; and (3) to develop consensus recommendations on genotoxicity testing around the world (Kirkland *et al.*, 2000). The main topics are (1) cytotoxicity in the *in vitro* chromosomal aberration test (Galloway, 2000); (2) *in vitro* micronucleus test (Kirsch-Volders *et al.*, 2000); (3) mouse lymphoma assay (Moore *et al.*, 2000); (4) photochemical genotoxicity assays (Gocke *et al.*, 2000); (5) comet assay (Tice *et al.*, 2000) (6) *in vivo* micronucleus test (Hayashi *et al.*, 2000); (7) transgenic mutation assays (Heddle *et al.*, 2000) (8) detection of DNA adducts (Phillips *et al.*, 2000) etc. The major conclusions are summarized by Lovell *et al.* (2000). In this article, I will introduce recent advanced toxicological methods to evaluate and to elucidate the mechanism of genetic toxicity and/or carcinogenesis.

## Single Cell Gel Electrophoresis (Comet) assay

As one of the mechanisms of carcinogenicity, induction of DNA damage was ascertained by a comet assay, which is widely used for the detection and measurement of DNA strand breaks. Since Ostling and Johanson (1984) introduced microelectrophoretic technique, Singh *et al.*

(1988) have modified and improved the microgel electrophoresis technique to evaluate DNA damage in single cells under alkaline conditions. The single cell gel electrophoresis (SCGE, comet or microgel electrophoresis) assay is rapid, sensitive, visual and simple technique to quantify DNA strand breaks in individual cells. It can be noticed that there are 3 major basic protocols: the first of Ostling and Johanson (1984), the second of Singh *et al.* (1988, 1990, 1991a,b, 1994, 1995a,b) and the one of Olive *et al.* (1993, 1994). Each protocol follows the principle of embedding cells in agarose, lysing the cytoplasmic material and exposing the remaining cell nuclei to a weak electric field. Large differences can be found in the physical and chemical conditions of each version of the method.

If the agent causes the strand breakage, we can see the extent of tail from the head (nucleus) shaped like comet with staining of fluorescent dyes such as ethidium bromide, acridine orange, propidium iodide and so on. However, there are some variations in procedure and conditions between laboratories a kind of cells used. So, to harmonize this variations in comet assay, International Workshop on Genotoxicity Test Procedure (IWGTP) was held at Washington D.C. on March, 1999 by Environmental Mutagen Society supported with OECD. Our laboratory (Ryu *et al.*, 1999c, 2001a, 2001e) also involved in this harmonization and published as preliminary form for OECD guideline with Tice *et al.* (2000). Comet assay is useful in various fields such as radiation, human monitoring in work place and toxicity evaluation of some chemicals etc. General reviews on this technique have been published by Tice *et al.* (1991, 2000) and Fairbairn *et al.* (1995, 1996).

## Cell culture and viability test

The mouse lymphoma cell line L5178Y (*tk*<sup>+/−</sup> 3.7.2c) was used for the experiment. Cells were cultivated in 90% RPMI-1640 (Life Technologies, MD, USA) with 1 mM sodium pyruvate, 0.1% pluronic supplemented with 10% heat-inactivated horse serum and antibiotics in a humidified incubator at 37°C with 5% CO<sub>2</sub>. For the determination of cell viability, about 10<sup>6</sup> cells were treated for 2 hr with test chemicals. BaP was used in combination with S9 mixture. After the staining of 0.4% trypan blue (Life Technologies, MD, U.S.A), the total number of cells and the number of unstained cells were counted in five of the major sections of a hemocytometer. The average number of cells per section

was calculated. Cell viability of treated groups was compared to solvent controls in the presence and absence of S9 mixture. All experiments were repeated twice independently.

#### Preparation of agarose coated glass slide

For the comet assay,  $8 \times 10^5$  cells were seeded into 12 wells plate (Falcon 3043) and then treated with test compounds. In the experiments with metabolic activation, parallel cultures were performed in the absence or presence of S9 mixture. After 2 min, cells were centrifuged for 5 min at  $100 \times g$  (about 1,200 rpm), and gently resuspended with PBS and 100  $\mu$ l of the cell suspension was immediately used for the test. Cells were mixed with 0.1 ml of 1% low melting point agarose (LMPA, Gibco BRL, Life Technologies, Inc., MD, USA) and added to fully frosted slide (Cat. No., 12-544-5, Fisher Scientific, PA, USA) which had been covered with a bottom layer of 100  $\mu$ l of 1% normal melting agarose (Amresco, OH, USA). The cell suspension was immediately covered with cover glass and the slides were then kept at 4°C for 5 min to allow solidification. After removing the cover glass gently, the slides were covered with a third layer of 100  $\mu$ l of 0.5% LMPA by using a cover glass and then the slide were kept again at 4°C for 5 min.

#### Alkaline unwinding and alkaline electrophoresis

The procedure follows the method described by Singh *et al.*, (1988) with minor modification. The cells embedded in the agarose on slides were lysed for 1.5 hr in reaction mixture of 2.5 M NaCl, 0.1 M Na<sub>2</sub>-EDTA, 10 mM Tris-HCl (pH 10), 1% Triton X-100 at 4°C. Slides were then placed in 0.3 M NaOH containing 1 mM Na<sub>2</sub>-EDTA (approximately pH 13) for 20 min to unwind DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

#### Evaluation and Statistics of DNA damage

After the electrophoresis, the slides were washed gently to remove alkali and detergents which would interfere with ethidium bromide staining, by placing horizontally and flooding them three times slowly with 0.4 M Tris (pH 7.5) for 5 min. The slides were stained by 50  $\mu$ l of ethidium bromide in distilled water solution on each slide, and then covering the slide with a cover

glass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed from each sample. All experiments were repeated in an independent test. Measurement was made by image analysis with Komet 3.1 (Kinetic Imaging Limited, Liverpool, UK) system, determining the mean tail moment (percentage of DNA in the tail times tail length) of the 50 cells. Differences between the control and the other values were tested for significance using one way of analysis of variance (ANOVA).

#### Mouse lymphoma thymidine kinase (tk)<sup>+/-</sup> gene assay (MOLY)

MOLY was performed with L5178Y tk<sup>+/-</sup> mouse lymphoma cells as described by Clements (1994) with minor modification (Clive *et al.*, 1983, 1995, Sawyer *et al.*, 1985; Garriott *et al.*, 1995; Oberly and Garriott, 1996; Sofuni *et al.*, 1996, Ryu *et al.*, 1999b). The cytotoxicity of chemical was determined by relative survival (RS) after 3 hr treatment at concentrations up to 5000  $\mu$ g/ml with and without S-9 mixture. The highest concentration was chosen one with a 10-20% RS. This experiment consisted of one solvent control, one positive control and at least three test chemical concentrations in duplicate cultures. Cultures were exposed to the test chemical for 3 hr, then cultured for 2 days before plating in 96-well microtiter plates at 2000 cells/well with trifluorothymidine for mutant selection and at 1.6 cells/well for cell viability. The number of wells containing colonies was counted on day 12 after plating, and large and small colonies were scored. Mutation frequencies were analyzed by the statistical package, Mutant V2.31 program (Hazleton, England) in accordance with the UKEMS guidelines. The acceptable ranges of mean absolute plating efficiency (PE) for solvent control were 60-140% for survival (PE0) and 70-130% for viability (PE2), based on the consensus agreements (Clive *et al.*, 1995).

#### Supravital staining *in vivo* micronucleus assay with peripheral reticulocytes

The micronucleus (MN) assay *in vivo* is a method devised primarily for screening chemicals for chromosome-breaking effects. In the monitoring of chromosome breakage, the assay is at least as sensitive as the metaphase method; in addition it includes effects on the

spindle apparatus. This properties render the assay highly suitable for routine toxicological screening. The method is based on the following principles and observations: in anaphase, acentric chromatid and chromosome fragments lag behind when the centric elements move towards the spindle poles. After telophase the undamaged chromosomes, as well as the centric fragments, give rise to regular daughter nuclei. The lagging elements are included in the daughter cells, too, but a considerable proportion is transformed into one or several secondary nuclei which are, as a rule, much smaller than the principal nucleus and are therefore called micronuclei.

The MN assay using peripheral blood erythrocytes was introduced by MacGregor *et al.* (1980) and developed by Hayashi *et al.* (1990, 1994b) into more simple and convenient method compared to conventional bone marrow assay by the introduction of supravital acridine orange staining. Giemsa staining has been routinely used for the MN assay. The Giemsa method, however, has some disadvantages because Giemsa stains not only MNs but also some cell inclusions containing RNA and other acidic materials dark blue; it is occasionally difficult to identify MNs from these inclusions. Acridine Orange (AO) metachromatic fluorochrome discriminates between DNA and RNA by green and red fluorescence, respectively. The method provides advantages that high-quality specimens can be easily obtained without special skill, and can be reduced intra- and inter-laboratory variation.

When this method is combined with other toxicological studies, chromosomal damage could be assessed together with other toxicological endpoints.

#### *In vitro* cytokinesis-block micronucleus assay

The study of DNA damage at the chromosome level is an essential part of genetic toxicology because chromosomal mutation is an important event in carcinogenesis. The micronucleus assays have emerged as one of the preferred methods for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably (Heddle *et al.*, 1983). Because micronuclei can only be expressed in cells that complete nuclear division a special method was developed that identifies such cells by their binucleate appearance when blocked from performing cytokinesis by cytochalasin-B, a microfilament-assembly inhibitor. The cytokinesis-block micronucleus (CBMN) assay allows

better precision because the data obtained are not confounded by altered cell division kinetics caused by cytotoxicity of agents tested or sub-optimal cell culture conditions. The method is now applied to various cell types for population monitoring of genetic damage, screening of chemicals for genotoxic potential and for specific purposes such as the prediction of the radiosensitivity of tumours (Shibamoto *et al.*, 1991) and the inter-individual variation in radiosensitivity. In its current basic form the CBMN assay can provide, using simple morphological criteria, the following measures of genotoxicity and cytotoxicity: chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), cell division inhibition, necrosis and apoptosis (Fenech *et al.*, 1999). The cytosine-arabioside modification of the CBMN assay allows for measurement of excision repairable lesions (Fenech *et al.*, 1994). The use of molecular probes enables chromosome loss to be distinguished from chromosome breakage and importantly non-disjunction in non-micronucleated binucleated cells can be efficiently measured (Zihno *et al.*, 1994). The *in vitro* CBMN technique, therefore, provides multiple and complementary measures of genotoxicity and cytotoxicity which can be achieved with relative ease within one system.

#### Transgenic Mutagenesis assay system

Because mutagens often produce characteristic patterns of DNA sequence alterations (Dogliotti, 1996), called mutation spectrum, the elucidation of mutation spectrum of specific carcinogen provides a basis for understanding of cancer etiology and action mechanism of chemical carcinogenesis (Gorelick, 1995).

Recently developed transgenic mutagenesis assay system including Big Blue mutagenesis system is a useful and powerful tool to evaluate the genotoxicity of chemicals, and it also provides a window of carcinogenesis and mutagenesis mechanisms of chemicals based on information such as mutation pattern, frequency, and location in sequence context of the *lacI* target gene (Gorelick, 1995). The *lacI* transgenic Big Blue Rat2 fibroblast cell line carries over 40 copies of lambda shuttle vector (Dycaico *et al.*, 1994) containing *lacI* gene as a target (Lundberg *et al.*, 1993; Heddle and Tao, 1995; Summers *et al.*, 1989). The *lacI* gene, as a mutational target, is very useful for the study of the mutational characteristics of a carcinogen for several

reasons. First, the relatively small size (1,080 bp of coding region) of *lacI* gene facilitates sequence analysis. Second, the expression of repressor protein permits a rapid colorimetric assay to screen for mutations. In addition, it is possible to make use of the large historical database in subsequent comparisons, allowing us to elucidate the underlying mechanisms leading to mutations (Kohler *et al.*, 1991). The mutations induced in the *lacI* gene can easily be quantified by mutant frequency (MF), and the precise mutation type and distribution can quickly be identified by direct sequencing. Moreover, considering that mutations in *lacI* gene induced by chemicals reflect the effects of mutagens on other endogenous genes such as proto-oncogenes and tumor suppressor genes, and that mutations occurred in these genes are the most common events in many types of human cancer (Kohler *et al.*, 1991; Tao *et al.*, 1993; Gossen *et al.*, 1989), this assay may provide a powerful tool to predict the mutation spectrum induced in cancer-related genes more accurately. Our laboratory elucidated mutation spectrum of 4-nitroquinoline N-oxide (Ryu *et al.*, 1998a,c, 1999a) and 1,2-dibromo-3-chloropropane (2001f, 2002). And also, we reported the mutant frequency of atrazine with cytogenetic analysis using Big Blue Rat2 cells (2000).

#### Cell culture

The *lacI* transgenic Big Blue Rat2 fibroblast cell line was purchased from Stratagene (La Jolla, CA). This cell line is derived from a Rat2 embryonic fibroblast cell line (CRL 1764; ATCC, Rockville, MD) transfected with the Big Blue shuttle vector (lambda *lacI* / *lacZ* shuttle vector) and pSV2NEO plasmid, which provides an antibiotic selection marker. The shuttle vector, which has been integrated into the Rat2 genome at two sites (~60 copies/cell), is identical to that used for generating the Big Blue mouse. The *lacI* transgenic Big Blue Rat2 fibroblast cells were cultured as a monolayer in Dulbeccos modified Eagles medium (DMEM; Gibco BRL, NY) containing 50 units/ml penicillin, 50 mg/ml streptomycin, 200 mg/ml geneticin (G418), and 10% heat-inactivated defined fetal bovine serum (Gibco BRL, NY) at 37°C in a humidified incubator containing 5% CO<sub>2</sub> (Ryu *et al.*, 1999a). Under these conditions, the average cell cycle time was ~19 hr. Low passage (<5) cells were used in each experiment. Cells were treated at 30-40% confluence (~3.5×10<sup>5</sup> cells in a 25 cm<sup>2</sup> flask). A solution of 0.25% trypsin-EDTA (Gibco BRL, NY) was used for subculture.

#### Transgenic mutagenesis assay

**a : Isolation of genomic DNA.** Genomic DNA was isolated from *lacI* transgenic Big Blue Rat2 fibroblast cells using the RecoverEase protocol (Stratagene, La Jolla, CA). In essence, cell pellets were thawed on ice, resuspended in 8 ml of ice-cold lysis buffer (8.20 g NaCl, 0.22 g KCl, 120 g sucrose, 0.30 g EDTA, 10 ml Triton X-100, 1.58 g Tris-HCl (pH 8.3)) and homogenized with a tissue grinder. The homogenate was filtered through a 100 µm nylon mesh filter into 50 ml tubes and centrifuged at 1,100×g for 12 min at 4°C. The supernatant was discarded and the tubes were swabbed with a cotton swab to remove residual supernatant. The pellet was incubated at 50°C for 45 min in the presence of 70 µl digestion buffer containing RNase-It ribonuclease cocktail (20 µl/ml digestion buffer; Stratagene, La Jolla, CA) and 70 µl proteinase K solution. The contents of the tube were dialyzed for 48 hr by placing the DNA extract on the wetted surface of a membrane floating on 10 mM Tris/1 mM EDTA buffer, pH 7.4. The viscous DNA was collected and stored at 4°C until packaging.

**b : Packaging and plating of DNA.** After DNA concentrations were adjusted to 0.5 mg/ml, the genomic DNA was incubated with Transpack packaging extract (Stratagene, La Jolla, CA) to excise the lambda vector target and package it into a lambda head according to the Stratagene Big Blue instruction manual. The titer of the rescued phage was estimated by plating the packaged phage in serial dilutions. For plating, the volume of the phage equivalent to 15,000 plaque forming units was added to 2 ml of *Escherichia coli* SCS-8 cells (Stratagene, La Jolla, CA) in 10 mM MgSO<sub>4</sub>. Pre-warmed Big Blue top agarose containing 1.5 mg/ml of X-gal (dissolved in dimethylformamide) was added, and the contents were poured onto 25×25 cm<sup>2</sup> assay trays containing 250 ml Big Blue bottom agar (agar, casein peptone, yeast extract, MgSO<sub>4</sub> and NaCl). Trays were incubated for 18 hr at 37°C and scored for blue mutant plaques. The total number of plaques in each plate was estimated by counting four sectors of 5×5 cm<sup>2</sup> areas, averaging these numbers and multiplying by a scaling factor. Blue mutant plaques were counted and picked into individual tubes containing 0.5 ml SM buffer (5.8 g NaCl, 2.0 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 50 ml 1 M Tris-HCl (pH 7.5) and 5 ml of 2% gelatin (W/V)) for further characterization. To confirm the mutant phenotype and for future use in DNA sequence analysis, all recovered putative mutant phages were diluted to 1:200 and replated on 100 mm

plates with 3.5 ml of top agarose containing 1.5 mg/ml X-gal. The sectorized plaques that were observed were also verified for their phenotype as previously specified and confirmed sectorized plaques were scored separately. The *lacI* mutant frequency (MF) value was calculated by dividing the number of verified mutant plaques by the total number of plaques analyzed.

#### Mutation spectrum

The *lacI* gene was sequenced from *lacI* mutants of solvent and medium control cells (collectively considered as spontaneous mutants) and of cells exposed to chemical. Each mutant was purified by replating on a 100 mm plate at low density. From each plate, a single mutant plaque was isolated, cored, placed into 25  $\mu$ l deionized water, and then boiled for 5 min. The *lacI* gene was amplified by PCR in 100  $\mu$ l PCR reaction mixture containing 15  $\mu$ l of phage DNA, 2  $\mu$ l of each of forward position 53 to 37, (5-CCCAGACACCATCGAATG-3) and reverse position 1,201 to 1,185, (5-ACCATTCC ACACAACATAC-3) Big Blue PCR primers, 25 mM dNTPs and 5 U *Ex Taq* polymerase (TaKaRa Shuzo Co. Ltd, Japan). After the initial denaturation step at 95°C for 5 min and extension step at 72°C for 5 min, 30 cycles of amplification were performed as follows: denaturation at 95°C for 90 s, annealing at 55°C for 90 s, elongation at 72°C for 150 s with a final extension at 72°C for 10 min using thermocycler (Robocycler 40 temperature cycler, Stratagene, La Jolla, CA). The resulting 1,300 bp fragment was purified with a PCR purification kit (Inje Biotech, Seoul, Korea) according to the manufacturers instructions, and visualized on a 1% low melting point (LMP) agarose gel containing 1 mg/ml ethidium bromide. The PCR products were sequenced with an ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer, CA) on an Applied Biosystem 373 DNA sequencer (Perkin-Elmer, CA). The purified DNA sample corresponding to approximately 200 ng was added to 3.2 pmol/ml primer and terminator dyes. The sequencing reactions were carried out 25 cycles as follows: 96°C for 30 s; 50°C for 15 s; 60°C for 4 min, according to the protocol established by Applied Biosystems.

#### Statistical analysis

Analysis of variance (ANOVA) test was performed to evaluate differences in *lacI* mutant frequency between treatment groups at the level of  $P = 0.05$ . A Dunnetts

multiple comparison test was used to compare each treatment group with untreated controls. Chi-square ( $\chi^2$ ) test was used for statistical treatment of mutational spectra.

### Toxicogenomics

The recent completion of the human genome sequencing project and the push to finish the mouse genome have raised the stakes in science with predictions of disease cures, more effective and safer pharmaceuticals, and a greater understanding of environmental effects on human health. The impact of human genome projects on toxicological research is high, heralding the emerging technologies of toxicogenomics, proteomics, and bioinformatics (Lovett, 2000, Pollack, 2000) for the future use of these technologies and their impact on drug discovery, safety evaluation, elucidation of pathways of toxicity, and risk assessment. The utilization of these new technologies along with more established genetic approaches such as quantitative polymerase chain reaction (PCR), and the use of genetically altered animals will dramatically move the field of toxicology forward.

High-throughput cDNA and oligonucleotide arrays and 2-D gel electrophoresis systems have quickly emerged as the premier tools to enable genomewide analysis of gene expression at the RNA and protein level. These new technologies are heavily influencing drug discovery and preclinical safety in the biotechnology and pharmaceutical industry (Freeman, 2000). Toxicologists are also promoting genomic expression technologies as a superior alternative to traditional rodent bioassays to identify and assess the safety of chemicals and drug candidates for human safety (Afshari *et al.*, 1999; Nuwaysir *et al.*, 1999; Pennie *et al.*, 2000). It is expected that gene expression profiling will identify mechanisms of action that underlie the potential toxicity of chemicals and drug candidates. Ultimately, toxicogenomics (the integration of genomics, bioinformatics, and toxicology) is expected to accelerate drug development and aid risk assessment. Recent experiments applied to cancer genetics have demonstrated the potential of gene expression profiling to accurately classify disease phenotypes (Alizadeh *et al.*, 2000; Bittner *et al.*, 2000), thus lending hope that expression profiling may classify and thus predict phenotypes of toxicity. Despite these expectations, it is still uncertain how gene expression profiling

experiments will ultimately contribute to our understanding of toxicity and allow us to realize the full potential of this new technology. Pennie *et al.* (2000) have also discussed the possibilities and caveats of gene expression profiling in the context of mechanistic and predictive toxicology and have addressed the certainty, biological relevance, and need for validation of microarray data.

There are both advantages and limitations to the use of gene array and proteomics technologies in toxicologic screening. The main advantage is a global approach to understanding the complex mechanisms involved in toxicology. Gene arrays have been costly and limited in availability, but the past year has shown a commitment by the scientific community to the general use and availability of gene arrays. Consequently, cost has been reduced by increased supply and demand. Furthermore, the availability and cost is substantially improving with many universities and research centers establishing genomic and proteomic facilities. Therefore, toxicogenomics and proteomics will certainly become generally used technologies in the near future.

Toxicogenomics and proteomics are also providing new biomarkers for use in human studies. These, coupled with the more traditional biological responses currently in use, provide a vast armamentarium for assessing exposures, effects and susceptibility factors relating to environmental pollutants. It is now possible to envision schemes for integrating the results of molecular epidemiological investigations into the general toxicological evaluations of environmental agents. These will allow intermediate endpoints to be used for making realistic human health assessments and for elucidating pathogenic mechanisms that identify targets for intervention, all with the goal of preventing environmentally mediated human disease. Finally, select biomarker responses that predict the likelihood of disease occurrence will find application in the interpretation of individual medical diagnostic tests, with the goal of improving cancer detection and management.

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