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Toxicity Assessment of Industrial Chemicals and Airborne Contaminants: Transition from *In Vivo* to *In Vitro* Test Methods: A Review

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Exposure to occupational and environmental contaminants is a major contributor to human health problems. Inhalation of gases, vapors, aerosols, and mixtures of these can cause a wide range of adverse health effects, ranging from simple irritation to systemic diseases. Despite significant achievements in the risk assessment of chemicals, the toxicological database, particularly for industrial chemicals, remains limited. Considering there are approximately 80,000 chemicals in commerce, and an extremely large number of chemical mixtures, *in vivo* testing of this large number is unachievable from both economical and practical perspectives. While *in vitro* methods are capable of rapidly providing toxicity information, regulatory agencies in general are still cautious about the replacement of whole-animal methods with new *in vitro* techniques. Although studying the toxic effects of inhaled chemicals is a complex subject, recent studies demonstrate that *in vitro* methods may have significant potential for assessing the toxicity of airborne contaminants. In this review, current toxicity test methods for risk evaluation of industrial chemicals and airborne contaminants are presented. To evaluate the potential applications of *in vitro* methods for studying respiratory toxicity, more recent models developed for toxicity testing of airborne contaminants are discussed.

Exposure to occupational and environmental contaminants is a major contributor to human health problems that can cause adverse effects ranging from simple irritation to morbidity and mortality due to acute intense or long-term, low-level repeated exposures (Klaassen, 2001; Greenberg et al., 2003; Winder & Stacey, 2004). Inhalation exposures can occur with gases, vapours, solid and liquid aerosols and mixtures of these. While evaluating the impact of chemicals on human health requires toxicity data, in many cases, particularly for industrial chemicals, the availability of toxicity information is quite limited (NTP, 1984; Agrawal & Winder, 1996; U.S. EPA, 1998; Faustman & Omenn, 2001; Marchant, 2005). The current approach of measuring the toxic effects of workplace contaminants relies on whole-animal test methods. As well as ethical concerns, heavy reliance on animal data in toxicology is the subject of debate and controversy by the scientific community (Blaaboer, 2002). Moreover, increasing the number of available industrial chemicals and new products has created a demand for alternatives to animal methods for better safety evaluation. Although studying the toxic effects of inhaled chemicals is technically more challenging, the potential application of *in vitro* methods to study the toxicity of airborne contaminants needs to be taken into consideration. In this review, methods available for investigating the toxic effects of industrial chemicals and airborne contaminants are addressed with a focus on the development, validation, and potential applications of *in vitro* test systems. Moreover, the more recent *in vitro* models for studying the toxic effects of inhaled chemicals are reviewed.

AIRBORNE CONTAMINANTS

Air contaminants are exogenous substances in indoor or outdoor air, including both particulates and gaseous contaminants that may cause adverse health effects in human or animals, affect plant life, and impact the global environment by changing the atmosphere of the earth (Raabe, 1999). Various physical, chemical, and dynamic processes may generate air pollution leading to emission of gases, particulates, or mixtures of these into the atmosphere (Lioy & Zhang, 1999). While great attempts have been made to reduce emissions from both stationary and mobile sources, millions of people today face excessive air pollution in

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both occupational and urban environments (Costa, 2001). Many industrial and commercial activities release toxic contaminants in gas, vapor, or particulate forms. Occupational and environmental health problems can potentially arise where such releases are not controlled properly. Moreover, today air contaminants are not limited to the urban environment or to the workplace but are also common indoor air contaminants present in office workplaces, schools, and hospitals.

TARGET ORGAN: RESPIRATORY SYSTEM

The major physiological function of the respiratory tract is the transfer of oxygen to the blood and removal of carbon dioxide as a metabolic waste. The human respiratory tract is ideally structured to achieve this function, given its very large surface area of approximately 140 m^2 and high daily exchange volume of more than 10 m^3 (Beckett, 1999; Boulet & Bowie, 1999; Hext, 2000; Witschi & Last, 2001). In addition, the membrane between air and blood in the gas exchange region is extremely thin, approximately 0.4–2.5 μm (Brouder & Tardif, 1998; Winder, 2004a). As well as olfactory, gas exchange, and blood oxygenation functions, the respiratory system has evolved to deal with xenobiotics and airborne materials such as those usually occurring in the air environment (Lambre et al., 1996). However, this system cannot always deal adequately with the wide range of airborne contaminants that may occur in urban and particularly occupational environments (Winder, 2004a). As a result, the respiratory system is both a site of toxicity for pulmonary toxicants, and a pathway for inhaled chemicals to reach other organs distant from the lungs and elicit their toxic effects at these extrapulmonary sites.

Responses to inhaled toxicants range from immediate reactions to long-term chronic effects, from specific impacts on single tissue to generalized systemic effects (David & Wagner, 1998; Winder, 2004b). The severity of toxic effects of inhaled chemicals is influenced by several factors such as type of air contaminant, airborne concentration, size of airborne chemical (for particles), solubility in tissue fluids, reactivity with tissue compounds, blood-gas partition coefficient (for gases and vapors), frequency and duration of exposure, interactions with other air toxicants, and individual immunological status (David & Wagner, 1998; Rozman & Klaassen, 2001; Winder, 2004b). The site of deposition/action of inhaled toxicants will determine, to a great extent, the ultimate response of the respiratory tract to inhaled chemicals. After inhalation, airborne contaminants may be deposited in different regions of the respiratory tract, including nasopharyngeal, tracheobronchial, and pulmonary regions.

DEPOSITION OF AIRBORNE CONTAMINANTS IN THE RESPIRATORY TRACT

Apart from the physiological and anatomical characteristics of the respiratory tract, physicochemical and aerodynamic characteristics of inhaled chemicals are crucial in determining the site of deposition/absorption and the ultimate fate of inhaled contaminants.

Gases and Vapors

Water solubility is the significant factor that determines the penetration site of a gas in the respiratory tract. For example ammonia, formaldehyde, sulfur dioxide, and hydrogen fluoride are extremely soluble in water and tend to be absorbed nearly completely within the nose and upper airways. While the nose acts as a scrubber for water-soluble gases to protect the lungs from potential toxic effects, the drawback of this protective action is the probability of production of toxic effects in the nose such as formaldehyde, which may induce nasal cancer (Rozman & Klaassen, 2001; IARC, 2004). Gases with low solubility, such as nitrogen dioxide, ozone, and phosgene, penetrate further into the pulmonary region and exert their toxicity in this region. Ultimately, very insoluble gases such as carbon monoxide and hydrogen sulfide pass through the respiratory tract efficiently and are delivered throughout the body through the pulmonary blood supply system. Although water solubility is a critical parameter in penetration of gaseous contaminants, other factors such as partition coefficient may also have a considerable influence. For example, when a gas penetrates to the gas-exchange region, the blood-gas partition coefficient will determine the rate of gas uptake into the blood (Hext, 2000; Rozman & Klassen, 2001).

Aerosols

Particle size distribution is the most critical characteristic that determines airborne behavior and deposition pattern of aerosols in the respiratory tract (Asgharian et al., 1995). Airborne particles usually are polydispersoid with an approximately log-normal size distribution. During inhalation, a specific volume of air from the breathing zone accelerates towards the nose (or mouth) and enters into the respiratory tract. As well as air, particles with specific median cut-point of aerodynamic diameter (d_{50}) that can follow the airstream will enter into the respiratory tract. Larger particles usually are deposited in the nasopharyngeal region by an inertial impaction mechanism (inhalable fraction, $d_{50} = 100$ μm). Aerosols that fail to be captured in the nasopharyngeal region will be deposited in the tracheobronchial region (thoracic fraction, $d_{50} = 10$ μm) and may be absorbed or be cleaned by mucociliary clearance. Finally, the remaining particles with the smallest size distribution that are not trapped in the tracheobronchial region will penetrate deeply into the alveolar region (respirable fraction, $d_{50} = 4$ μm) of the respiratory tract and diffuse to respiratory tissues (ACGIH, 2001).

TOXICITY ASSESSMENT APPROACHES

While data obtained from human experiences would be most useful in assessing the toxic effects of chemicals, human data is not always available for developing safety evaluations on chemicals and airborne contaminants. Moreover, because of unfortunate human experiences such as those with pharmaceutical

agents like diethylstilbestrol and thalidomide, or occupational and environmental contaminants like lead and polychlorinated biphenyls (PCBs), it is now understood that the risks of chemicals, new products, and technologies need to be assessed before adverse human experiences occur (McClellan, 1999; Thorne, 2001; Greenberg & Philips, 2003). Therefore, as part of preventive strategies, it is critical to develop new approaches that are both informative and time/cost efficient to identify the potential hazards in the absence of widespread human exposures (Silbergeld, 1998). In general, no single method can cover the complexity of general toxicity in humans (Barile, 1994). However, toxicity data can be obtained from several sources, including toxicological studies, epidemiological studies, quantitative structure–activity relationships (QSARs), and physiologically based toxicokinetic (PBTK) studies.

Toxicology has made a major contribution in providing chemical toxicity information for many years. Today, toxicology continues to develop by the application of the risk assessment process as a systemic, scientific approach for characterizing and quantifying potential adverse effects of chemicals (Faustman & Omenn, 2001). Moreover, *in vitro* toxicology methods, using cell culture technology in combination with knowledge of structure–activity relationships and toxicokinetics, are currently being developed and implemented in modern toxicology. From a methodology perspective, toxicological test methods ranging from conventional whole animals usage (*in vivo*) to modern cell culture (*in vitro*) techniques.

In Vivo Toxicity Test Methods

Conventional methods of toxicological assessment are based on whole-animal studies. A basic assumption of *in vivo* or animal toxicity studies is: Chemicals that cause adverse effects in animals can cause such effects in humans as well. Thus, data obtained from animals can be extrapolated to humans by a variety of techniques. Animal toxicity studies began in 1927 when the LD_{50} test was introduced by Trevan in the United States (Trevan, 1927). As the LD_{50} can provide essential information on the toxic effects of chemicals, this test usually is performed on a new chemical in order to identify acute oral toxicity. The process involves the feeding to animals of a test chemical at increasing doses until the dose that is capable for killing 50% of test animals is determined (OECD, 2001a). It is necessary that the LD_{50} values indicate the route of exposure (oral or dermal), the animal species used in the test, and the unit of measurement of dose (usually mg/kg body weight) (Cote et al., 1998).

Acute toxicity data are usually applied to toxicity classification and labeling requirements for risk assessment of chemical substances. More recently, a globally harmonized system (GHS) of classification has been proposed for physicochemical, single- or repeated-dose toxicity and environmental toxicity endpoints (United Nations [UN], 2003). In this classification, criteria have been developed for a range of different single dose toxicity endpoints. Based on acute toxicity by the oral, dermal and inhalation route, chemicals include one of five escalating toxicity categories according to the numeric criteria expressed as LD_{50} (oral, dermal) or LC_{50} (inhalation) values (OECD, 2001b; UN, 2003). It is anticipated that when implemented the GHS will enhance the protection of human health and the environment under a globally harmonized system of classification.

In recent years, conventional animal toxicity tests, particularly tests such as the LD_{50}, are criticized on ethical grounds by animal welfare groups due to the possible use of large numbers of animals and the suffering such tests can cause. Hence the Organization for Economic and Cooperative Development (OECD) has developed three alternative tests for acute oral toxicity, the Fixed Dose Procedure (420), the Acute Toxic Class Method (423), and the Up and Down Procedure (425), to modify the conventional acute oral toxicity test (401) in order to reduce the number of animals used per test substance (OECD, 2001a). Besides these ethical concerns, heavy reliance on animal data in toxicology has long been a concern of the scientific community. Prediction of biological activities of toxic compounds in humans with reliance on animal data always poses some degree of uncertainty due to interspecies differences between animals and humans (O'Hare & Atterwill, 1995; Gad, 2000; Blaaboer, 2002). Even within a species, great interindividual variation may occur due to more subtle genetic variations (intraspecies differences), (Faustman & Omenn, 2001). Moreover, the validity of extrapolation from high doses/concentrations to real-world low-level exposures is being questioned, particularly when such extrapolations are not supported by mechanistic information (Eisenbrand et al., 2002). Although the magnitude of a toxic effect is usually proportional to the exposure level, the biological differences between high- and low-dose scenarios needs to be considered (Benford et al., 2000). Toxicologists have traditionally attempted to overcome these uncertainties by introducing uncertainty factors in establishing safety standards for human exposures (Faustman & Omenn, 2001; Eisenbrand et al., 2002).

*Respiratory Toxicity Study **in vivo***

Extensive background data have been developed from toxicological studies using animal models. However, most of these studies are conducted by oral and dermal exposures rather than inhalation exposure (Agrawal & Winder, 1996; Miller & Klonne, 1997). While toxicology data may exist from other routes of exposure, the extrapolation of these data is most difficult to validate. In order to identify the lethal effects of air toxicants, inhalation toxicity tests need to be carried out in test animals. Test animals are exposed to air toxicants dissolved or suspended in air, contaminated air is inhaled, and the concentration that causes lethality in 50% of dosed group (LC_{50}) is determined. In reporting an LC_{50}, both the concentration of the chemical in air that can cause death in 50% of exposed animals and the time of exposure should be indicated. Standard protocols have been adopted by regulatory agencies for both short-term and long-term inhalation tests. The OECD has initially adopted guidelines for acute inhalation toxicity (403), repeated-dose inhalation

toxicity 28/14-day (412), and subchronic inhalation toxicity 90-day (413). Meanwhile, new guidelines for acute inhalation toxicity, such as the Acute Inhalation Toxicity-Fixed Dose Procedure (433) and Acute Inhalation Toxicity-Acute Toxic Class (ATC) Method (436), are being finalized (OECD, 2004).

In inhalation studies, evaluating the dose received by the animal is more challenging due to several factors that influence the actual dose, such as atmospheric concentration, duration of exposure, pulmonary physiological characteristics of the test animal, and deposition/absorption patterns of the air contaminant. In a specified system for many inhaled chemicals the actual dose is related to the product of concentration (c) and exposure time (t), which is known as Harber's law ($c \times t =$ inhaled dose) (Hext, 2000; Valentine & Kennedy, 2001). In many cases, particularly in short-time exposures to chemicals with direct action on the respiratory system, the response is directly proportional to the product of $c \times t$. However, many other substances, such as chemicals that exhibit systemic toxicity, do not follow Harber's law because in such cases several factors, including absorption by the respiratory tract, tissue distribution, biotransformation in potential target organs, and elimination, will influence the toxicity (Hext, 2000). Although Harber's law does not apply to all chemicals, it can be applied when extrapolated to $c \times t$ values that differ by a factor of about 3 to 4 for a given $c \times t$ product (Valentine & Kennedy, 2001).

The selection of an animal species for toxicity studies is another crucial consideration that may influence the outcome of *in vivo* studies and the estimated human adverse health effects. Usually rodents such as rat, mouse, guinea pig, and hamster are used. However, different criteria should be considered in the selection of animals, such as species-related physiological factors, the size of the animals, the availability of the animals, the number of animals needed, the cost of obtaining and maintaining required number of animals, and the cost of producing consistent atmospheric test concentrations (Valentine & Kennedy, 2001).

Generation and characterization of known concentrations of air contaminants and reproducible exposure conditions is a more complicated and expensive procedure than that required for oral and dermal exposures. This process requires different equipment and techniques to generate, maintain and measure standard test atmospheres. Inhalation exposure systems involve several efficient and precise subsystems, including a conditioned air supply system, a suitable gas or aerosol generator for test chemical, an atmosphere dilution and delivery system, an exposure chamber, a real-time monitoring or sampling and analytical system, and an exhaust/filter or scrubbing system (Phalen, 1997; Hext, 2000; Valentine & Kennedy, 2001).

The more common exposure modes to evaluate the effect of inhaled air toxicants involve whole-body, head-only, and nose-only. Whole-body exposure mode is conducted most frequently for human inhalation studies. Head-only exposure mode is suitable for repeated short-time exposure for restricting the portal of entry of the test chemical to the respiratory system. Design and construction of head-only units are similar to those of nose-only units. Nose-only exposure units require an animal holder to accommodate rodents with suitable size to reduce stress and discomfort. The animal holder is normally designed to fit the general shape of the animal, with a conical headpiece using polymethyl methacrylate, polycarbonate, or stainless steel. The animal holder is connected to the exposure chamber that introduces the test chemical into the face or nose of the animal.

Exposure chambers can be operated in both static and dynamic systems. Usually animal inhalation toxicity studies are carried out in a dynamic system to avoid particle settling and exhaled gas complications. While the operation of a static system is relatively simple and requires comparatively less test material, this is only suitable for short exposure times due to reliance on the air inside the chamber. In contrast, in a dynamic system, the test atmosphere flows through the exposure chamber continuously and hence ensures atmospheric stability and no reduction in oxygen concentration due to test animal respiration (Hext, 2000). This system requires accurate flow monitoring for both diluent air and primary source of contaminant in proportions that can produce the final desired concentrations.

In Vitro Toxicity Test Methods

The focus of toxicology has shifted somewhat, since the mid 1980s, from whole-animal toxicity tests to alternative *in vitro* toxicity methods (Silbergeld, 1998; Gad, 2000). Basic cell culture techniques are designed to provide a proper support of cells or tissues for their normal survival, growth, and function in vitreous media (formerly glass and more recently plastic) outside the body. Application of cell culture techniques in toxicological studies is referred to as *in vitro* toxicology, which describes a field of study that applies technology using isolated organs, tissues, and cell culture to study the toxic effects of chemicals (Hayes & Markovic, 1999). Since the first mammalian cells were grown in capillary glass tubes in the United States, cell culture technology, and hence *in vitro* toxicology, has progressed and during the last two decades gained an increasing acceptance in toxicological investigations (Barile, 1994; Gad, 2000). As well as scientific advances, the development of *in vitro* toxicity test methods has been influenced by a variety of socioeconomical factors.

Historical Perspective

The concept of alternatives, which is considered as the development of test systems other than those relying on whole animals, was started with the contribution of two British scientists, W. M. S. Russel and R. L. Burch, by the introduction of the three Rs in their book *The Principles of Humane Experimental Techniques* in 1959. The three Rs provide the conceptual basis for reconsideration of using animals in research and refer to reduction in the number of test animals, refinement of test protocols in order to minimize suffering of test animals, and replacement of current animal tests with appropriate *in vitro* tests. Initially, the principles introduced by Russel and Burch did not receive

attention until the growth of the animal welfare movement in the mid 1970s (Reinhardt, 1994; Silbergeld, 1998).

However, the development of *in vitro* test systems has been influenced by a number of factors (Purchase et al., 1998). Animal welfare issues are among the most important social concerns that have impacted on the recent shift toward alternatives in toxicity testing. Animal rights activities and increasing public awareness of certain animal testing requirements have forced researchers and regulatory agencies to increase and diversify the development and validation of alternative methods. Another social issue is the increasing public interest on the safety of chemicals and new products. Each year, thousands of new cosmetics, pharmaceuticals, pesticides, and consumer products are introduced into the marketplace. Considering there are approximately 80,000 chemicals in commerce (NTP, 2001), as well as the extremely large number of chemical mixtures, *in vivo* testing of these numbers of chemicals requires a large number of expensive, time-consuming, and in some cases nonhumane tests in animal species. The necessity of determination of the potential toxic effects of this large number of chemicals has provoked the need for rapid, sensitive, and specific test methods.

Applications of In Vitro Toxicity Methods

In vitro toxicity methods were first developed to study the mechanisms of actions of chemical substances at molecular and cellular level (Frazier, 1992). Increasingly, *in vitro* methods have been applied in many fields such as cancer biology, drug discovery, and toxicology. Genotoxicity was the first field of toxicology in which *in vitro* test methods have been used for toxicity testing to identify the mutagenic characteristics of chemicals (Ames et al., 1973, 1975). At present, in vitro methods cover a broad range of techniques and models, and a standardized battery of *in vitro* tests can be used for assessing acute local and systemic toxicity. Cytotoxicity testing, reproductive toxicity, mutagenicity, irritancy testing, immunology, and target organ toxicity are the main areas of *in vitro* toxicology (O'Hare & Atterwill, 1995).

Meanwhile, the application of *in vitro* methods in toxicology is also being faced by a number of difficulties. One important shortcoming of *in vitro* methods is the lack of complexity (Zucco et al., 1998). While cells in culture represent the elementary living systems, as a very simplified system, they cannot represent the complexity of the entire organism. An *in vitro* system cannot replicate exactly the biodynamics of the whole human body due to the lack of possible mitigating systems (such as hormones, nervous system, and immunity), and the lack of biotransformation and excretion pathways for their elimination *in vitro* (Hayes & Markovic, 2002). The challenge of how to relate concentrations that produce cellular toxicity *in vitro* to equivalent *in vivo* dosages may be improved by the development and application of predictive tools such as physiologically based toxicokinetic (PBTK) models (Frazier, 1992). The basic toxicology knowledge of absorption, distribution, metabolism, and elimination (ADME) of chemicals is essential for the development of appropriate toxicokinetic models. The next limitation of these methods is related to chronic toxicity testing. Although some studies are promising (Scheers et al., 2001), to date, *in vitro* test systems are mainly focused on acute toxicity testing rather than short-term or long-term repeated dose toxicity investigations (Eisenbrand et al., 2002). More knowledge of the mechanisms of toxicity is still needed before *in vitro* methods could broadly be implemented for repeated dose toxicity testing (Lambre et al., 1996). Physicochemical properties of test chemicals such as low water solubility or high vapor pressure may also cause technical problems during the course of *in vitro* tests (Stark et al., 1986; Frazier & Bradlaw, 1989; Ciapetti, 1998).

Nevertheless, *in vitro* toxicity test systems offer new advantages when compared to traditional *in vivo* toxicity methods (Balls & Fentem, 1992; Frazier, 1992; Anderson & Russell, 1995; Zucco et al., 1998, 2004; Eisenbrand et al., 2002; Holme & Dybing, 2002). In vitro test methods eliminate the interspecies extrapolation by using human cells and tissues, which may assist to generate more representative data for human toxicological risk assessments. These methods are comparatively simpler, faster, and hence less time-consuming and more cost-efficient (Anderson & Russel, 1995). They facilitate the application of biochemical, cellular, and molecular biology techniques to study the underlying mechanism of toxic action (Blaauboer, 2002; Holme & Dybing, 2002). In vitro models for organ toxicity evaluation developed for many organs and tissues (such as nephrotoxic, neurotoxic, and hematotoxic models) allow the study of components of potential target organs and target systems (Zucco et al., 2004). Despite the complex organization of cells, *in vitro* methods are also developed for respiratory toxicity assessment using relevant airway cells, lung cells, or tissues and implementation of target specific endpoints (Lambre et al., 1996; ICCVAM, 2001).

Considering these advantages, *in vitro* toxicology is one of the most rapidly expanding areas of alternative methods with many practical applications (Barile, 1994; Faustman & Omenn, 2001; Eisenbrand et al., 2002). *In vitro* toxicity methods can be used for screening, ranking, and risk assessment of chemicals and new products. These methods can be applied to determine the adverse effects of industrial chemicals, pharmaceuticals, and consumer products that are being manufactured and marketed at rapid and extraordinary rates (Barile, 1997). Many of these products have never been adequately tested, mainly due to the cost of animal tests (Marchant, 2005). Further, these chemical entities are formulated into hundreds of thousands of chemical products, where not only is the toxicity of individual ingredients poorly understood, but the potential toxic interactions between individual chemicals in such mixtures are unpredicted (Yang, 1994). The potential toxic interactions of chemical mixtures can be assessed using *in vitro* test methods (Malich et al., 1998; Cross et al., 2001).

Mechanistic information provided by *in vitro* toxicity methods has both theoretical and practical applications. Such information can supply a scientific base for interpretation of descriptive toxicity data and assessment of the possibility that a

chemical will induce adverse effects if those effects occur at the molecular or cellular level. Mechanistic approaches may facilitate to explore procedures to prevent or antagonize toxicity, formulate new drugs and industrial products that are less harmful, and design pesticides that are more selectively toxic for their target organisms (Silbergeld, 1998; Faustman & Omenn, 2001). For example, the application of pharmacogenetics and pharmacogenomics in the drug development process may not only improve the safety and efficacy of new drugs but also improve the development of optimal dosing regimens (Lesko et al., 2003). In addition, *in vitro* methods can be applied as biomarkers of chemical hazards such as biomonitoring of food, water, and air for environmental and occupational toxicants (Barile, 1994; Eisenbrand et al., 2002). In occupational and environmental toxicology biomarkers have a potential to be used as a predictive tool via biomonitoring of the causal pathway between exposure and effect. This pathway may be illuminated by intelligent application of new molecular biology technologies such as genomics (DNA sequences) and proteomics (protein expression) (Benford et al., 2000).

In Vitro Toxicity Endpoints

Different levels of organization in the human body may be affected by chemical substances from molecules to cell to tissue to organ to functional levels. Cytotoxicity is the adverse effects that occur from the interaction of a toxicant with structures and/or processes essential for cell survival, proliferation, and/or function (Ekwall, 1983). Toxic chemicals can attack any of the basal cell functions, origin-specific cell functions, and extracellular functions. To assess the potential cytotoxicity of chemical substances, several *in vitro* tests have been developed by measuring different biological endpoints, which are summarized in Table 1 (Balls & Clothier, 1992; Barile, 1994, 1997; Anderson & Russell, 1995; Zucco et al., 1998; Doyle & Griffiths, 2000; Freshney, 2000; Wilson, 2000). In addition, over recent years, research on apoptosis has enormously changed the knowledge of mechanisms involved in cell death, leading to the development of mechanistically based endpoints. Many morphological and biological changes that may occur at the cellular membrane, nucleus, specific proteases, and DNA level can be used as biological endpoints for measuring apoptosis (Wilson, 2000; Zucco et al., 2004). Moreover, the rapid progress in genomic, transcriptomic (gene expression), and proteomic technologies has created a unique powerful tool in toxicological investigations (Eisenbrand et al., 2002).

TABLE 1
Common biological endpoints assessed by in vitro toxicity tests

Biological endpoint	Detection method
Cell morphology	Cell size and shape
	Cell–cell contacts
	Nuclear number, size, shape, and inclusions
	Nuclear or cytoplasmic vacuolation
Cell viability	Trypan blue dye exclusion
	Diacetyl fluorescein uptake
	Cell counting
	Replating efficiency
Cell metabolism	Mitochondrial integrity (MTT, MTS, and XTT tetrazolium salt assays)
	Lysosome and Golgi body activity (neutral red uptake)
	Cofactor depletion (e.g. ATP content)
Membrane leakage	Loss of enzymes (e.g., LDH), ions or cofactors (e.g., Ca^{2+}, K^{+}, NADPH)
	Leakage of prelabeled markers (e.g., ^{51}Cr or fluorescein)
Cell proliferation	Cell counting
	Total protein content (e.g., methylene blue, Coomassie blue, Kenacid blue)
	DNA content (e.g., Hoechst 33342)
	Colony formation
Cell adhesion	Attachment to culture surface
	Detachment from culture surface
	Cell–cell adhesion
Radioisotope incorporation	Thymidine incorporation into DNA
	Uridine incorporation into RNA
	Amino acids incorporation into proteins

Validation of In Vitro Test Methods

Currently, *in vitro* toxicity methods have gained different values with regard to reliability and acceptance. While some procedures may be directly substituted in place of existing animal models, many other procedures are used for screening or in adjunct tests (Reinhardt, 1994; Gad, 2000). For instance, one of the longest standing validated and regulatory accepted alternatives is the *Limulus* amebocyte lysate (LAL) test for detection of pyrogenic endotoxins in pharmaceutical products as a replacement to the rabbit pyrogen test (Liebsch, 1995). In addition, presently, a few well-defined instances such as skin corrosivity (OECD *in vitro* skin corrosion tests; 430 and 431) and phototoxicity (OECD *in vitro* 3T3 NRU phototoxicity test; 432) tests have successfully been validated and accepted for regulatory purposes (Spielmann et al., 1998; Spielmann & Liebsch, 2001; Carere et al., 2002; Indans, 2002; Liebsch & Spielmann, 2002). A number of prevalidation studies for regulatory purposes are currently in progress by the European Centre for Validation of Alternative Methods (ECVAM), such as neurotoxicity, nephrotoxicity, and carcinogenicity testing (Carere et al., 2002). Development and validation of new alternative methods that offer additional or more accurate toxicity information can strengthen the science base and hence persuade their adoption by regulatory authorities (NTP, 2001). Although traditional animal test methods are unlikely to be eliminated and substituted at present, every effort should be encouraged to

develop and validate alternative test systems. As Professor Balls (2004) predicted, changes will occur but not rapidly: "Progress will be made only if both time and money are invested in the search for alternatives. Changes will occur step by step, with each step being carefully evaluated before it is accepted and implemented. Unfortunately, there are no instantaneous solutions" (p. 167).

The current status of *in vitro* methods for assessing acute toxicity has been reviewed in a workshop organized by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and it has been concluded that while animals are currently used for the evaluation of acute toxicity, recent correlation studies demonstrate that *in vitro* methods may also have significant potential in prediction of human acute toxicity (ICCVAM, 2001; Botham, 2004). Moreover, as the ultimate goal of toxicity tests is the prediction of toxicity in humans, the development and validation of human cell-based methods are preferred. After this evaluation study, a collaborative study on the use of basal cytotoxicity tests for estimating both rodent LD_{50} values and human lethal concentrations has been initiated by the ICCVAM and ECVAM. In this study, a total 72 reference chemicals are currently being tested using normal human keratinocyte (NHK) cells and BALB/*c* 3T3 fibroblasts in three different laboratories (Botham, 2004). One outcome of this validation study may be the introduction of *in vitro* test results in the field of regulatory toxicology as an alternative to those from animal studies.

To characterize the current status of the application of *in vitro* cytotoxicity assays in the prediction of acute toxicity *in vivo*, a number of approaches have been reviewed by the ICCVAM workshop. The two main approaches included the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) and the German Center for the Documentation and Validation of Alternative Methods (ZEBET) approaches (ICCVAM, 2001).

ZEBET approach. The ZEBET approach is proposed by the German Centre for the Documentation and Validation of Alternative Methods. In this approach, data from RC (Registry of Cytotoxicity), which is a database of acute oral LD_{50} values from rat and mice taken from the NIOSH Registry of Toxic Effects of Chemical Substances (RTECS), and 50% inhibitory concentration (IC_{50}) values of chemicals from *in vitro* cytotoxicity assays are used. The RC database contains a regression analysis of *in vitro* IC_{50} values and *in vivo* rodent LD_{50} values for 347 chemicals. Basal cytotoxicity appeared to be an acceptable predictor of the LD_{50} for 74% of the RC chemicals (Halle & Spielmann, 1992; ICCVAM, 2001). Therefore, it is expected that most chemicals with acute toxicity may reflect an insult to the basal intrinsic cell functions (Eisenbrand et al., 2002). Based on the ZEBET proposed strategy, *in vitro* cytotoxicity data, in the short term, can be used to predict the starting dose for *in vivo* testing in order to reduce the use of animals (Halle & Spielmann, 1992; ICCVAM, 2001).

MEIC program. The MEIC cytotoxicity program, organized by the Scandinavian Society for Cell Toxicology (SSCT), was an international research project to investigate the relevance of *in vitro* toxicity assays for the prediction of human acute toxicity. In this study, carried out between 1989 and 1996, 50 reference chemicals, selected from different classes of chemicals, were tested in 97 laboratories using 61 *in vitro* methods. To provide a background to the *in vitro*/*in vivo* evaluation, rodent LD_{50} values were correlated with human lethal doses, indicating a relatively good correlation ($R^2 = .61$ and $R^2 = .65$) for the rat and mouse respectively (Ekwall, 1999). To investigate the relevance of *in vitro* findings, IC_{50} values from *in vitro* assays were correlated with human lethal blood concentrations (LCs) complied from human poisoning reports. Human cell line assays were determined as the best predictor of human toxicity ($R^2 = .74$) when compared to animal cell lines ($R^2 = .64$), (Ekwall, 1999). One important outcome of the MEIC study was that a test battery of *in vitro* assays can be used directly to predict human acute lethal toxicity (Ekwall, 1999). Therefore, once disposition information (absorption, biotransformation, elimination) is taken into account, *in vitro* assays have the potential to be used as a surrogate for single-dose toxicity, as usually obtained from LD_{50} and similar animal toxicity tests. The results of the MEIC study are provided as a series of papers in *Alternative To Laboratory Animals (ATLA) Journal*, and overviews of the final MEIC results have also been published (Clemedson & Ekwall, 1999; Ekwall, 1999).

Respiratory Toxicity Study In Vitro

The study of the toxic effects of inhaled chemicals is typically more challenging due to the technology required for the generation and characterization of test atmospheres, and the development of effective and reproducible techniques for exposure of cell cultures to airborne contaminants. A practical approach for *in vitro* respiratory toxicity testing has been proposed by ECVAM (Lambre et al., 1996). This systemic approach is initiated with the consultation of existing literature, evaluating the physicochemical characteristics of test chemicals, and predicting potential toxic effects based on structure–activity relationships (SARs). Physicochemical characteristics of chemicals such as molecular structure, solubility, vapor pressure, pH sensitivity, electrophilicity, and chemical reactivity are important properties that may provide critical information for hazard identification and toxicity prediction (Gad, 2000; Faustman & Omenn, 2001).

Initial *in vitro* tests should be conducted to identify likely target cells and toxic potency of test chemicals. Based on the obtained result, *in vitro* tests may be followed by a second phase using the following cells: nasal olfactory cells, airway epithelial cells, type II cells, alveolar macrophages, vascular endothelial cells, fibroblasts, and mesothelial cells (Lambre et al., 1996). Moreover, advantages and limitations of using different cell types and endpoints for toxicity measurement of air contaminants have been discussed in this workshop. While more than 10 main cell types have been identified in the epithelium of the respiratory tract, for the assessment of respiratory toxicity it is

important to utilize specific cell types with appropriate metabolizing activity. Therefore, freshly isolated cells maintained in suitable culture media that can mimic biotransformation activities and cellular functions comparable to the *in vivo* environment are preferred to long-term cultures or cell lines that may differentiate, lose their organ-specific functions, and lack the enzyme systems required for biotransformation (Barile, 1994; Lambre et al., 1996). It has been suggested the endpoints to be used need to be selected based on the knowledge of toxic effects of test chemicals and should always include cell viability testing in at least two different cell types. A better understanding of mechanisms involved in respiratory toxicity and also the development of standardized and reproducible *in vitro* exposure and delivery systems that simulate inhalation exposure *in vivo* were encouraged by the ECVAM workshop.

The toxic effects of air contaminants have been studied using several *in vitro* exposure techniques (Table 2). Most of these, especially studies conducted on particulates, are limited to exposure of cells to test chemicals solubilized or suspended in culture medium (Nadeau et al., 1995; Governa et al., 1997; Goegan et al., 1998; Baeza-Squiban et al., 1999; Becker et al., 2002; Takano et al., 2002; Riley et al., 2003; Okeson et al., 2004). Although this exposure condition may be adequate for soluble test materials, this may not follow the *in vivo* exposure pattern of airborne aerosols, particularly for insoluble aerosols, due to unexpected alternation of their compositions and particle–media or particle–cell interactions (Diabate et al., 2002). Moreover, such techniques for exposure may often ignore size, which is crucial in toxicity testing of inhaled particles. Some other researchers have employed sampling the aerosols on filters followed by the investigation of the effects of suspended and extracted particles, such as those studies that have been conducted on atmospheric aerosols (Hamers et al., 2000; Yamaguchi & Yamazaki, 2001; Alfaro Moreno et al., 2002; Glowala et al., 2002; Baulig et al., 2003) or cigarette smoke condensate (Bombick et al., 1998a, 1998b; McKarns et al., 2000; Putnam et al., 2002). Filtration offers an advantage for on site toxicity assessments of aerosols; however, this technique usually requires sample preparation steps, such as extraction to isolate the components of interest from a sample matrix, and ultimately, solubilization in culture media, potentially increasing experimental errors and further toxicity interactions.

TABLE 2
In vitro exposure techniques developed for toxicity study of air contaminants

Exposure technique	Exposure achievement procedure
Exposure to test chemical itself	Cells are exposed to test chemicals solubilized or suspended in culture media
Exposure to collected air samples	Cells are exposed to suspended or extracted particulates collected by filtration technique
Submerged exposure condition	Test gas is introduced to cell suspension under submerged condition using impinger or vacuum test tubes
Intermittent exposure	Cells are periodically exposed to gaseous compound and culture medium at regular intervals using variation of techniques: rocker platforms, rolling bottles
Continuous direct exposure	Cells are continuously exposed to airborne contaminants during the exposure time, usually on their apical side, while being nourished from their basolateral side, using collagen-coated or porous membranes permeable to culture media

Several *in vitro* models have also been developed to deal with gas-phase exposure of airborne contaminants using different exposure techniques. Different features of exposure techniques developed for gaseous compounds have been discussed in terms of their relevance, advantages, and limitations (Ritter et al., 2001). In principle, these methods include exposure of cells under submerged conditions, intermittent exposure procedures, and more recently direct exposure techniques.

A submerged exposure condition can easily be achieved using variation of laboratory procedures to introduce test gaseous compounds to cell suspensions. For example, in order to study the *in vitro* toxic effects of ozone on human haematic mononucleated cells (HHMC), the test gas was introduced at once to cell suspensions in vacuum test tubes (Cardile et al., 1995). However, exposure patterns *in vivo* may not be closely simulated and only a very small interface between the test gas and the target cells can be provided by the submerged exposure technique.

Variation of laboratory techniques have been developed allowing intermittent exposure of cultures to gaseous contaminants. Cell culture dishes held on chambers or platforms rounded, shacked or tilted at certain angles were exposed to gaseous compounds periodically (Van Der Zee et al., 1987; Blanquart et al., 1995; Rusznak et al., 1996). Cell culture flasks were also tilted at regular intervals to expose the cell cultures to volatile anaesthetics (Muckter et al., 1998). Rolling culture bottles on roller drums were set up for *in vitro* gas exposure (Shiraishi et al., 1986). Lung slices were alternatively fed by culture medium and exposed to the diesel exhausts by rotating the culture vial on the internal wall of a flow-through chamber (Morin et al., 1999). Tissue culture flasks on a rocking platform were used to expose the cells to mainstream cigarette smoke, followed by an immersion in culture media intermittently (Bombick et al., 1998c). Compared to submerged exposure, the intermittent exposure technique provides a larger interface between gaseous compound and target cells. Nevertheless, in such exposure conditions cells are always covered by an intervening

layer of medium that may influence both accuracy and reproducibility of the results.

More recently, technology has become available that allows cells to be cultured on permeable porous membranes in transwell or snapwell inserts (Suppliers; Costar, Falcon and Nunc). Once cells are established on the membrane, the upper layer of culture media can be removed, and the cells are directly exposed to air contaminants. In a direct exposure technique at the air/liquid interface target cells can be exposed to airborne contaminants continuously during the exposure time on their apical side, while being nourished from their basolateral side. Direct exposure of cells to airborne contaminants was initially achieved by growing cells on collagen-coated membrane located on special constructions (Chen et al., 1993) and more recently porous membranes in transwell inserts (Knebel et al., 1998; Diabate et al., 2002; Auftherheide et al., 2003).

The toxic effects of sulfuric acid aerosols on human airway epithelial cells (Chen et al., 1993) and the effects of ozone on cultures of primate bronchial epithelial cells (Jabbour et al., 1998) or human bronchial cells (Mogel et al., 1998) have been studied using cells grown on collagen-coated membranes. Toxic effects of individual airborne chemicals such as ozone and nitrogen dioxide (Knebel et al., 1998) and complex mixtures such as diesel motor exhaust (Knebel et al., 2002) and cigarette smoke (Aufderheide et al., 2003) were studied using cultured human lung cells on porous membranes permeable to culture media. Direct exposure technique at the air/liquid interface offers a reproducible contact between chemically and physically unmodified airborne contaminants and target cells and technically may reflect more closely inhalation exposure *in vivo* (Ritter et al., 2001; Diabate et al., 2002; Knebel et al., 2002; Aufderheide et al., 2003). A typical experimental set up for air/liquid exposure requires appropriate exposure chambers. Standard tissue culture incubators are used (Lang et al., 1998) or specific exposure chambers (Aufderheide & Mohr, 2000) are designed for this purpose.

CONCLUSION

Environmental and occupational exposure to chemical contaminants is a major health concern. While animals have been traditionally used to study the toxic effects of chemicals and inhaled contaminants, recent studies demonstrate that *in vitro* methods may also have significant potential for studying respiratory toxicity. Development, standardization, and validation of reproducible *in vitro* test methods could play a significant role in the study of respiratory toxicity and may lead to a better understanding of the interactions between chemical exposure and toxic effects. The most appropriate cell systems with biotransformation activities and cellular functions comparable to the *in vivo* environment, such as primary cell cultures and human cell-based assay systems, need to be implemented.

Although *in vitro* toxicology methods cannot exactly mimic the biodynamics of the whole body, *in vitro* test systems in combination with the knowledge of quantitative structure–activity relationships (QSARs) and physiologically based toxicokinetic (PBTK) models have a potential to be considered more broadly for risk assessment of human chemical exposures. A systemic approach for respiratory toxicity should commence with a comprehensive review of existing data, assessment of the physicochemical properties of test chemicals, and employment of any structure–activity relationships (Lambre et al., 1996). A key molecular structure may provide some readily available information on toxicity prediction (Faustman & Omenn, 2001). Further, the application of physiologically based toxicokinetic models may provide a scientific basis for extrapolation of *in vitro* concentrations which produce cellular toxicity *in vitro*, to equivalent *in vivo* dosages.

The study of the toxic effects of inhaled chemicals *in vitro* requires the development of effective and reproducible techniques for exposure of cell cultures to airborne contaminants. In this article, *in vitro* methods developed for toxicity testing of air contaminants have been reviewed and their advantages and limitations in toxicity testing of inhaled chemicals have been discussed. Direct exposure technique at the air/liquid interface offers the possibility of exposing human target cells to physically and chemically unmodified airborne contaminants. During the exposure time, continuous direct exposure can be achieved by growing cells on porous membranes in transwell or snapwell inserts. Direct exposure techniques may have the potential to be applied extensively to study the toxic effects of airborne contaminants and respiratory toxicity in vitro, as the exposure pattern *in vivo* is more closely simulated by this method. Further, development of appropriate *in vitro* sampling and exposure techniques may provide an advanced technology for biomonitoring of occupational and environmental airborne contaminants.

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