13th Meeting of the Scientific Group on Methodologies for the Safety Evaluation of Chemicals (SGOMSEC): Alternative Testing Methodologies for Organ Toxicity

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In the past decade *in vitro* tests have been developed that represent a range of anatomic structure from perfused whole organs to subcellular fractions. To assess the use of *in vitro* tests for toxicity testing, we describe and evaluate the current status of organotypic cultures for the major target organs of toxic agents. This includes liver, kidney, neural tissue, the hematopoietic system, the immune system, reproductive organs, and the endocrine system. The second part of this report reviews the application of *in vitro* culture systems to organ specific toxicity and evaluates the application of these systems both in industry for safety assessment and in government for regulatory purposes. Members of the working group (WG) felt that access to high-quality human material is essential for better use of *in vitro* organ and tissue cultures in the risk assessment process. Therefore, research should focus on improving culture techniques that will allow better preservation of human material. The WG felt that it is also important to develop and make available relevant reference compounds for toxicity assessment in each organ system, to organize and make available via the Internet complete *in vivo* toxicity data, including human data, containing dose, end points, and toxicokinetics. The WG also recommended that research should be supported to identify and to validate biological end points for target organ toxicity to be used in alternative toxicity testing strategies. — *Environ Health Perspect* 106(Suppl 2):427–439(1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-2/427-439spielmann/abstract.html*

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Abbreviations used: ATP, adenosine triphosphate; CFU-GM, colony-forming unit–granulocyte/macrophage; CYPs, cytochrome P450s; ECVAM, European Centre for the Validation of Alternative Methods; EPO, erythropoieten; ES cell, embryonic stem cells; IC_{90}, inhibitory concentration 90; LLNA, local lymph node assay; NK, natural Killer; OECD, Organisation for Economic Co-operation and Development; PELs, permissible exposure limits; RT-PCR, reverse transcriptase–polymerase chain reaction; SAR, structure–activity relationships; SHBG, sex hormone-binding globulin; WG, working group; UDS, unscheduled DNA synthesis.

Introduction

Detecting specific organ toxicity is critical to toxicity testing. Current *in vivo* tests can in fact do so, but these tests are expensive, time consuming, and animal intensive. For the past decade toxicologists have been developing alternative *in vitro* tests for assessing organ toxicity. Indeed, *in vitro* tests have been developed that represent a range of anatomic structures from perfused whole organs to subcellular fractions.

Table 1 details the various preparations used to assess toxicity of the major organs along with a listing of limitations and advantages of the systems. Many of these can be prepared from a variety of animals including humans. Indeed many human tissues are readily available, i.e., blood and other fluids, liver, kidney, etc. When possible, human tissue experiments should be given highest priority.

The most physiological organ preparation for toxicity studies is the isolated perfused organ. This system maintains tissue architecture that allows for cell–cell interactions and measurement of organ secretory/excretory activity. However, the main disadvantages are that it does not reduce the number of animals used and there is significant variability between animals.

The next level of tissue organization available for toxicity testing is tissue slices. These have been successfully prepared from liver, heart, kidney, and brain, to name a few. Although tissue architecture is maintained, cell preservation is problematic. Isolated suspended cell preparations are used for blood cells, including cells of the immune system. This works well for short-term studies.

The first part of this report updates the current status of organotypic cultures for the major target organs of toxic agents. This includes liver, kidney, neural tissue, the hematopoietic system, the immune system, reproductive organs, and the endocrine system. The second part reviews the application of *in vitro* culture systems to organ-specific toxicity and evaluates future prospects.

Current Methods for Organotypic Cultures

Liver

In vitro models of liver toxicity are detailed by Guillozo (*1*). The liver is a major target organ for toxic compounds. Hepatotoxicity can be predictable or can be idiosyncratic

Table 1. Advantages and limitations of *in vitro* preparations.

Model	Advantages	Limitations
Isolated perfused organ (liver, heart, lung, kidney, spleen, gonad)	Three-dimensional architecture preserved Cell–cell interactions preserved (between all resident cell types) Histological examination possible No influence of higher order systems (humoral, endocrine, nervous, etc.) Only *in vitro* model for secretory/excretory collection Maintenance of epithelial vascular interactions	Loss of organ functions [loss of interactions between distinct organs, including metabolic activation (except liver) cytokine interaction and endocrine regulation] Short-term studies (2–4 hr maximum) Demanding experimental technology (temperature, perfusate, oxygenation) One organ per experiment (animal consuming) Limited amount of compound and concentrations can be tested per experiment Human organs normally not available All models are hypoxic if perfused (erythrocyte-polymerized hemoglobin) Standardization difficult (high-cost equipment and consumables)
Tissue slices, 0.25 mm[a] (liver, heart, lung, kidney, skeletal muscle, brain, etc.)	Tissue architecture preserved Cell–cell interactions preserved Histological examination possible Human material available for some organs (liver, lung, kidney, brain) Studies on several chemicals at different concentrations possible Interspecies comparison possible	Only available for short-term studies (from a few hours to 2–3 days) Not all the cells in slices, dependent on function, cell types, and location within the section Further loss of organ functions (secretory/excretory functions)
Isolated suspended cells[a,b]	Various types from various tissues available, for example, free cells (blood cells) or freshly isolated cells (e.g., immune cells) Usually the cells retain tissue-specific characteristics (receptors, metabolism) Studies of several chemicals at different concentrations possible Cryopreservation Interspecies and interindividual comparison possible	Isolation method not for all tissues and not standardized Only for short-term studies (few hours) Further loss of organ functions (loss of polarity and intercellular signaling for certain cell types)
Primary cell cultures and early subcultures[a,b,c]	Survival: at least a few days or a few passages Retainment of differentiated functions under appropriate culture conditions Several chemicals can be tested at different concentrations Interspecies and interindividual variability can be assessed Coculture possible with other cell types (generation of extracellular matrix, improvement of differentiation) Induction studies possible (transcription/translation) Permits three-dimensional cultures (spheroids, tubules, cysts)	Further loss of organ function (phenotypic changes and rapid loss of the most differentiated functions) Unstable phenotype Some functions cannot be analyzed (e.g., bile excretion in liver primary cultures)
Cell lines[a,c]	Immortalization of primary cells (e.g., with SV 40, papilloma virus) Unlimited number of cells can be obtained Appropriate for only some specific mechanistic studies Interspecies studies are limited Genetic engineering, namely, transfection of defined genes possible (e.g., P450, transport proteins, receptors) Cryopreservation	No match to *in vivo* correspondents due to loss of specific functions (enzymes, transport proteins, receptors) Unstable genotype Further loss of cell function (unstable genotype and loss of specific functions, resistant to anticancer drug toxicity)
Subcellular fractions S9 fraction	Drug-metabolizing enzyme activities preserved Interspecies studies possible	Short-term studies (30–60 min) Loss of receptor proteins
Microsomes	Interspecies studies possible Covalent binding studies Drug-metabolizing enzyme activities preserved	Cytosolic enzymes missing Short-term studies (30–60 min)
Mitochondria	ATP synthesis β-Oxidation of fatty acids Oxidative phosphorylation	Technically demanding Short-term studies Often cannot be recovered from cultured cells
Vesicles of different plasma membrane domains	Receptor and transport studies	Loss of glycolipid anchored proteins
Genetically engineered cells (yeast, bacteria, insect cells, mammalian cells)	Express one or more human genes (CYPs, GST in development) Could replace S9 or liver microsomes in the Ames test Potentially many genes can be transfected stably Unlimited number of transfected cells	Currently still under development

GST, glutathione *S*-transferase. [a]Growth and/or incubation can be strictly controlled by using defined media (hormones, growth factors, etc.). [b]*In vitro* system is amenable to all organs. [c]Organotypic function can be more easily achieved by replacing static culture conditions by medium perfusion and use of selected growth supports (e.g., microporous membranes, hollow fibers, or carriers).

because of infrequent metabolic or immunologic pathways within an exposed population (*2*). The liver contains a variety of cell types, including hepatocytes, Kupffer cells, and ductal cells. Hepatocytes represent two-thirds of the total hepatic cell population and show a heterogeneous intralobular distribution of drug-metabolizing enzymes; these are more abundant in perivenous cells. The liver is richly endowed with phase 1 and phase 2 enzymes, mostly located in hepatocytes, with less activity in bile duct cells and some perhaps in Kupffer cells (*3*). More than 1000 chemicals are potentially hepatotoxic, and some of these require bioactivation by liver enzymes to become hepatotoxic. The liver can also produce metabolites that will be toxic for other organs or tissues. Various factors can modulate enzyme activities, e.g., age, gender, diseases, previous drug exposure, nutritional status, and genetic polymorphism. Species differences exist in drug-metabolizing enzymes, particularly between humans and laboratory animals, making extrapolation of animal data to humans imprecise (*1–3*).

Liver tissue and cells can be studied *ex vivo* in a variety of ways, each with specific advantages and limitations: isolated perfused organ, tissue slices, suspended cells, primary cultures, cell lines, subcellular fractions, and genetically engineered cells (Table 2) (*1*).

Because the isolated organ model is difficult to handle and cannot be used for human studies, tissue slices and isolated hepatocytes, either in suspension or in primary culture, are currently the most powerful models (*4,5*). They reflect the *in vivo* situation, at least for a few hours, and can be obtained from various species including humans and fish (*1,2*).

Hepatocyte survival and function in primary culture depend on adequate cell culture conditions, and they can also be stimulated to divide 1 to 2 times. Hepatocytes can be cocultured with other nonparenchymal cells: for example, with Kupffer cells to study the effects of cytokines or with stellate cells to study fibrogenesis. Hepatocytes cocultured with primitive biliary cells represent the most powerful model for long-term survival and function of hepatocytes (*6–10*). However, it must be underscored that no culture condition can maintain fully differentiated hepatocytes (some drug-metabolizing enzymes are rapidly lost, even if they remain inducible), and no hepatic cell line expresses the large spectrum of liver functions.

Human cytochromes P450 (CYP) can be expressed over short periods of time in several genetically engineered cells (yeast, bacteria, insect cells, mammalian cells). Liver S9 preparations and microsomes are used for the Ames test and oxidative metabolism studies. Finally, nonparenchymal cells can be isolated and cultured, e.g., Kupffer, endothelial, stellate, and bile duct cells (*1,2,9,10*).

Kidney

The kidney plays a central role in maintenance of body homeostasis, namely, water and electrolyte balance. Many aspects of renal structure and function render the organ especially susceptible to toxic xenobiotics. These include the high rate of

Table 2. Advantages and limitations of *in vitro* liver preparations.

Model	Advantages	Limitations
Isolated perfused liver	Functions close to those of the *in vivo* organ (all enzyme equipment preserved) Lobular structure preserved Functional bile canaliculi Collection of bile possible Short-term kinetic studies (extraction)	Short-term viability (2–3 hr) Study of one compound only Bile excretion decreased after 1–3 hr No study on human liver Suitable only for liver of small animals
Liver slices	Lobular structure preserved (all enzyme equipment preserved) Selective intralobular effects detectable Studies on human liver possible Studies on several compounds at different concentrations	Viability: 6 hr–2 days No collection of bile possible Not all the cells preserved similary (interassay variability)
Isolated hepatocytes[a]	Obtained from whole livers or wedge biopsies Functions close to those of *in vivo* hepatocytes Studies on several compounds at different concentrations Cryopreservation Interspecies studies Representative of the different lobular subpopulations	Short-term viability (2–4 hr) No bile canaliculus
Primary hepatocyte cultures[a]	Functions expressed for several days in certain conditions Induction/inhibition of drug-metabolizing enzymes Interspecies studies	Early phenotypic changes Altered bile canaliculi
Liver cell lines	Unlimited cell number Some functions preserved	Various drug enzyme activities lost or decreased Genotype instability
Subcellular fractions		
S9000 × *g* fraction	Drug enzyme activities preserved	Short-term studies
Microsomes	Drug enzyme activities preserved Production of metabolites for structural analysis	Short-term studies No cytosolic phase 2 enzyme reactions Cofactors required for activity
Mitochondria	ATP synthesis β-oxidation of fatty acids Oxidative phosphorylation	Short-term studies Cofactors required for activity
Genetically engineered cells	One or more human enzymes expressed Available only for CYPs Unlimited cell number	Use for specific purposes only No physiologic levels of enzymes

[a]Other cell types can also be isolated, cultured, and even cocultured with hepatocytes: for example, Kupffer, endothelial, stellate, and bile duct cells.

blood flow, the well-developed transport systems for solutes and ions, the capability to recover water and thereby concentrate the solutes to be excreted, and the dependency of viability upon high mitochondrial output. These tasks are confined to the epithelial cells resembling the renal functional units, the nephrons, which amount to 70 to 80% of total renal mass. A number of biochemical properties confined to epithelial cells are related to enzymes that may bioactivate drugs and other xenobiotics to become toxins. Activity of these enzymes is influenced by a number of factors (disease, drug, exposure, age). In addition immunoallergic mechanisms frequently induce renal toxicity. Considerable interspecies differences exist with respect to the induction of toxic nephropathies.

The isolated perfused (rat) kidney is most appropriate for studying potentially nephrotoxic xenobiotics when tubulovascular integrity is required (*11*). The advantage of the system is that no extrarenal regulatory influences (hormones, bloodborne factors) interfere in the study. This model enables precise control of the concentrations of compounds being studied. Because of its sophistication, it is, however, not suitable for routine application. It is an animal-consuming experimental system.

The isolated perfused nephron is not suitable for routine investigations, but this system provides data on enzyme localization and receptor distribution, which can be used for confirming the site of origin of isolated cells (*12*).

Renal slices have been used extensively in the past for renal transport and toxicity studies, typically for no more than a 2-hr period (*13*). Recently, precision-cut slices have been used. They are easy to produce, and commercial availability of slicers has helped to minimize interlaboratory variability. An advantage of slices is that they provide a multicellular system in which three-dimensional structures, and therefore cell–cell contacts, are preserved. Kidney-specific parameters are maintained and rapid and simple interspecies comparisons can be performed. Site-specific effects can be studied and several functional parameters can be assessed. The major disadvantages are limited lifespan and their morphological, functional, and biochemical heterogeneity. Collapse of nephron lumina hampers adequate transport studies. Slice surfaces always represent a region of tissue injury.

Isolated glomeruli and tubular fragments can be found in dissociated renal tissue fragments rich in proximal, distal, or collecting duct portions, or intact, decapsulated glomeruli. These fragments are viable and can be used for short periods to assess acute effects of chemicals. They have played a key role in showing that some nephrotoxicants (e.g., mercuric chloride) adversely effect glomeruli well before tubular damage occurs. Isolated renal proximal and distal tubular cells have been used extensively for acute nephrotoxicity studies. As they retain most of the characteristics seen *in vivo*, they can be used to study mechanistic aspects of toxicity at the cellular level and to design strategies for cytoprotection. They also may be used for *in vitro–in vivo* extrapolation and interspecies comparison. This model is hampered by the loss of cell viability, polarity, and junctional complexes after a maximum of 6 hr. These losses make the study of specific epithelial functions impossible. Approaches for establishing primary cultures of renal cells have been described by several laboratories (*14*). Because of the heterogeneity of renal tissue zones and nephron segments, it is essential to apply isolation procedures and selective culture conditions appropriate for cells of the nephron segment of interest. This *in vitro* system retains a number of the characteristics of renal cells *in vivo*. They form well-polarized epithelial monolayers and may retain adequate function over longer periods of time than in the models outlined above. For successful exploration of the effects of chemicals, the following requirements must be met: the cells must *a*) be polarized and possess intact junctional complexes, *b*) display vectorial transport of water and solutes and the appropriate uptake of xenobiotics, and *c*) express nephron cell-specific functions of transport, metabolism, and response to extracellular signals (e.g., hormones). Most but not all of these criteria can be met during the early phase of culture. However, cells tend to lose differentiated *in vivo* functions over time and may therefore not be useful for studying toxic side effects over prolonged periods. Inadequate culture conditions may accelerate the loss of this differentiated phenotype, but the use of defined media may help to stabilize the differentiated phenotype.

Continuous (immortalized) cell lines have been derived from certain nephron segments (Table 3). They are not transformed and have retained a number of renal epithelial characteristics, although they do not fulfill all the criteria listed above. Permanent cell lines keep their state of differentiation and can be used for studies of specific nephrotoxic mechanisms over prolonged periods up to several days or longer.

Table 3. Continuous renal epithelial cell lines.

Cell line	Species	Segmental origin	ATCC No.
LLC-PK1	Pig	Proximal tubule	ATCC CRL 1392
OK	Opossum	Proximal tubule	ATTC CRL 1840
JTC-12	Monkey	Proximal tubule	Not listed
MDCK	Dog	Distal tubule/ collecting duct	ATTC CCL 34
A6	*Xenopus laevis*	Distal tubule/ collecting duct	ATTC CCL 102

ATCC, American Type Culture Collection.

Neural Tissue

Neurotoxicity can be defined as any "adverse effect on the chemistry, structure and function of the nervous system, during development or at maturity, induced by chemical or physical influences" (*15*). Though controversy exists about the interpretation of the word adverse and the significance of reversible versus irreversible changes, there is agreement that changes such as neuropathy or axonopathy are definitely adverse neurotoxic effects. Standard acute, subacute, subchronic, and chronic toxicity studies are relevant to the assessment of potential neurotoxicity, as they are conducted at different dose levels, in different animal species, and with different durations of exposure. Clinical observations and morphological examinations from these studies can readily reveal effects on the nervous system. Specific neurotoxicity testing is conducted when there are indications of neurotoxicity, or on the basis of structure–activity or other considerations. This includes a series of observations, measurements, and neuropathological examinations in laboratory animals (*16–18*). Positive results in these Tier 2 studies would provide the basis for further tests that may include neurochemical or electrophysiological experiments, and are aimed at characterizing neurotoxic effects and identifying possible mechanisms. Special considerations, and additional testing, should be given to organophosphorus compounds (for their ability to cause delayed neuropathy) and to developmental neurotoxicants. As in other areas of toxicology, the desire to reduce the number of animals, and the time and costs of testing, has led to exploration of the possible use of *in vitro* approaches for neurotoxicity testing.

Several *in vitro* systems can be considered as alternative testing systems for

neurotoxicity (*19–22*). In decreasing order of complexity these models include organotypic explants, brain slices, reaggregate cultures, primary cell preparations, and established cell lines. Each model system has advantages and disadvantages. For example, all are derived from animals (rats or chicken) with the exception of cell lines and, in some occasions, primary cultures. In some systems, for example, organotypic explants or brain slices, the cytoarchitecture of the nervous system or certain neuronal circuitries or biochemical processes are preserved. On the other hand, primary cultures or cell lines allow the study of the effects of toxicants on isolated cell types (e.g., neurons, astrocytes, oligodendrocytes). Cell lines are usually the simplest to manipulate, but present the problem of being transformed, and may potentially display altered responsiveness (e.g., resistance) to toxicants. Systems that involve coculture of cells are also available, such as cocultures of neurons and astrocytes or oligodendrocytes, or astrocytes and endothelial cells, to mimic *in vitro* the blood–brain barrier (*23*).

Hematopoietic System

Leukocytes (granulocytes, monocytes, lymphocytes), erythrocytes, and platelets circulate in the blood where they perform specialized functions essential in immunity, oxygen delivery, and blood clotting. A hematotoxicant is defined as an external substance that causes a clinically significant adverse effect on the level or function of these cells (*24–28*). Decreased cell number is known as cytopenia and increased cell number cytosis. Blood cells are produced by precursor cells called progenitors, which are found in the bone marrow (and spleen of some animals). *In vivo*, hematotoxicants act by direct effects on blood cells or their progenitors, or by indirect effects mediated by humoral factors (*24–28*). The availability of recombinant cytokines has stimulated the development of *in vitro* assays for many progenitor populations in the myeloid, erythroid, platelet, lymphoid, and stromal lineages (*26*), and these progenitors have been cocultured with hepatocytes or transgenic cells to study bioactivation (e.g., via CYP) of protoxicants (*25*). Clonogenic assay for the neutrophil monocyte progenitor called colony forming unit–granulocyte/macrophage (CFU-GM) has been most commonly applied by laboratories in academia, industry, and government to the study of hematotoxicants in several species, including human (*25–27*), so SGOMSEC 13 focused on the evaluation of direct effects of toxicants on hematopoietic progenitors (*28*). Direct effects of toxicants on circulating blood cells (e.g., hemolysis) have been routinely assessed *ex vivo* and are not covered in detail in this workshop.

Immune System

In vitro models suitable for study of immune system toxicity are detailed by Karol (*29,30*). The immune system is a multicellular, multiorgan complex that includes the spleen, thymus, lymph nodes, and tonsils, as well as lymphoid areas of the gut and lung. The function of the immune system is to protect against agents such as bacteria, viruses, and particulates in the external environment, as well as against the internal development of "nonself" neoplasms. Dysfunction of the immune system is recognized as either heightened immune reactivity, as in hypersensitivity disease, or reduced immune surveillance that is manifested as reduced ability to combat infectious agents or tumors. Lymphoid cells from the blood, lymph nodes, and spleen, and fixed immune cells from solid tissues can all be isolated and studied *ex vivo*. Some of these cells can proliferate in response to added growth factors, whereas others are terminally differentiated and their specialized functions can be assessed.

Reproductive Organs

Reproduction is a continuous cycle, but for the purpose of toxicity testing it is divided into pregnancy, including prenatal and postnatal developmental toxicity, and the remainder of the reproductive cycle in both males and females when fertility may be impaired. To evaluate the reproductive toxicity of chemicals to humans, investigators conducted multi-generational studies in laboratory animals to provide information on the effects of industrial chemicals on all aspects of the highly complex reproductive cycle (*31*). In drug development, segment studies are conducted covering important phases of pre- and postnatal development as well as fertility (*32*). Because the complexity of the reproductive cycle and because of the lack of validated alternative tests for most of the steps in the cycle, testing in living animals is the only option currently available for assessing the possible effects of chemicals on reproduction.

To study fertility *in vitro*, methods for culturing ovarian and testicular cells and tissues both from laboratory animals and humans are established. Moreover, *in vitro* fertilization and embryo transfer, including the production of transgenic animals, are currently used worldwide in laboratory animals, farm animals, and for most of the techniques, also in humans. Therefore, techniques to assess the viability of sperm and oocytes to fertilize are in clinical use for both animals and humans (*33*).

Ex vivo cultivation of whole embryos is used to evaluate toxic effects on prenatal development in mammals, including humans. From fertilization to implantation, mammalian and human embryos can routinely be cultured. For the common rodent species, fairly simple whole-embryo culture techniques have been developed that usually start at the primitive streak stage and allow development to the most important phases of organogenesis. The usual culture period during which there is acceptable progress in development is usually 24 hr or less (*34*).

The development and differentiation of the most important embryonic organs can be studied in culture, for example, development of the limbs, heart, teeth, thyroid gland, central and peripheral neural tissue, and closure of the palatel shelves. The induction of embryonic tissues by other tissues, including induction of somites, the spinal cord and other organs, as well as interactions among tissues and the effects of growth factors and hormones, can be studied in specially designed systems, for example, transfilter systems (*33,34*).

Cells of almost all embryonic organs of mammals and most vertebrates can be maintained in primary culture. To study the developmental potential of embryonic cells of organs of specific interest, micromass cultures and aggregation cultures have proven to be very useful. Embryonic stem (ES) cells, which have the potential to differentiate into all tissues of the body, from laboratory animals and also from some farm animals, are routinely used to produce transgenic animals. ES cells of the mouse will differentiate under appropriate conditions into differentiated, mature cells, e.g., contracting cardiac cells (*35*). Embryonal carcinoma cells and permanent cell lines derived from embryonic organs of various mammalian species have been used in the past for many purposes. As they have usually lost specific characteristics of the organ from which they were derived, data obtained with such cells should be interpreted with caution. Like cell lines used in neurotoxicology, the malignant nature of these cells may confer unusual mechanisms of toxicant resistance that are not present in normal human tissues.

In conclusion, tissue culture systems to study the maturation of both oocytes and sperm, and also *in vitro* fertilization and preimplantation development, are adequate for toxicology studies. *In vitro* culture of mammalian embryos after implantation can only be studied for limited time periods. Standardization and improvement of culture conditions is recommended. The differentiation of ES cells is a promising area for understanding normal differentiation of undifferentiated pluripotent cells into highly specified cells. Improvement and standardization of ES cell culture conditions may allow better understanding of the mechanism of action of embryotoxic/teratogenic agents (*34,35*).

Endocrine System

Environmental endocrine disruptors have been defined as exogenous agents that interfere *in vivo* with the production, release, transport, metabolism, binding, action or elimination of natural ligands in the body responsible for the maintenance of homeostasis and the regulation of developmental processes. Consequently, targets within this system include behavior, synthesis and metabolism (i.e., aromatase), transport (i.e., sex hormone-binding globulin, transthyretin), steroid receptors (e.g., estrogen, androgen), and steroid-responsive genes as well as membrane receptors. It is conceivable that substances may be found that disrupt any or all of the protein, peptide, or amino acid endocrine or paracrine regulators.

Consequently, measurement of dysfunction of the endocrine system due to an environmental agent is similar in magnitude and complexity to the assessment of the nervous or immune systems, in that the endocrine system can affect the function and activity of multiple organs and cells at numerous sites and is under the constant control of feedback mechanisms. Thus, it is impossible to conceive that a single assay will be able to assess all of the potential interactions that may lead to endocrine disruption.

Currently, there are few established methods for the identification and assessment of chemicals and complex mixtures that elicit endocrine-disrupting activities. However, a number of *in vivo* and *in vitro* assays have been used to determine drug efficacy, or in basic research, to elucidate mechanisms of action. As a result, many of these assays have been adopted to predict the risk that endocrine disruptors pose to human health. At this point, there are no gold standard validated assays that have been accepted for regulatory purposes. Indeed, regulatory agencies as well as the scientific community are struggling to develop assays to screen for endocrine disruptors. A number of *in vivo* and *in vitro* assays have been proposed; their advantages and limitations have been recently reviewed and a compendium of existing methods for assessing the endocrine system has also been compiled (*36–38*). These assays examine a number of different end points and use a variety of species as well as mammalian cells in culture and yeast. However, in each case, there is a lack of standardized protocols and data assessment criteria. Therefore there are no established benchmarks or response parameters to ensure that the assays are performing optimally and to systematically compare the value of established or emerging assays.

Current Use of Test Systems. The identification and assessment of endocrine disruptors is complex as the elicited effects may be species-, ligand-, organ/tissue-, cell-, and response-specific. Moreover, the diversity of end points and the complexity of feedback mechanisms make a comprehensive assessment of the impact of an endocrine disruptor on endocrine function a difficult task. Therefore, to examine the potential endocrine-disrupting activities of a substance, a battery of *in vivo* and *in vitro* assays is needed.

Established *in vivo* methods for assessing the endocrine-disrupting activities of chemicals and complex mixtures include many of the parameters used in conventional reproduction/developmental studies. These studies are expensive and time consuming and their use is limited. Consequently, the challenge is to identify those substances that warrant such intensive investigation.

In vivo assays for endocrine disruptors are seriously lacking and require development. Vaginal cell cornification and effects on uterine wet weight performed in rodents are typically used to assess *in vivo* estrogenic activity (*37*). A number of different protocols and species have been used. These assays require standardized operating procedures (SOPs) that specify species, strain, age, and route of administration of test compound as well as other potential interventions (i.e., ovariectomy). Although these assays are considered to be the most established and accepted end points for *in vivo* estrogenic responses, they lack sensitivity and their appropriateness is questionable as rodents do not express sex hormone-binding globulin (SHBG) after parturition (*39*). Short-term *in vivo* assays for other endocrine disruptors (i.e., androgens, goitrogens) are essentially nonexistent and require intensive development.

In addition, endocrine disruptors may also elicit effects that are not manifested at the tissue or organ level. Consequently, it is essential that their effects be investigated at more sensitive end points such as at the level of gene expression. Assessment may be further complicated by the tissue-specific activities of some endocrine disruptors. For example, tamoxifen, a therapeutic antiestrogen used in estrogen responder positive breast cancer management, exhibits agonist activity in bone and the uterus but antagonist activity in mammary tissue. It apparently does not affect behavior.

Therefore, it is essential to measure a number of different genes since the elicited effects may be ligand- and response-specific. Gene expression can be accurately measured using sensitive and quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) assays that can also provide valuable data for risk assessment purposes.

A number of *in vitro* assays, namely competitive ligand binding, protein expression and enzyme activities, have also been used to investigate alleged endocrine disruptors. As with *in vivo* assays, *in vitro* assays also require the establishment of SOPs. Although *in vitro* assays exhibit greater sensitivity and selectivity and provide more information regarding potential mechanisms of action of endocrine disruptors, their use is also limited. *In vitro* assays possess minimal metabolic activity and cannot account for potential pharmacodynamic interactions. Moreover, because of the complexity of the endocrine system, it is not feasible to establish one assay that would be capable of predicting all of the potential adverse *in vivo* responses that may be elicited after exposure to an endocrine disruptor. Therefore, the assessment of an endocrine disruptor should include a battery of *in vitro* assays evaluating a number of different endocrine end points.

Emerging Methods. Several *in vivo* and *in vitro* methods are currently being developed that may assist in the identification and assessment of chemicals and complex mixtures that elicit endocrine-disrupting activities. Many of these assays are mechanistically based and take advantage of the known mechanisms of action of steroid hormones. However, these assays lack SOPs and they require further development to determine if they are predictive of *in vivo* responses. Emerging *in vitro* assays

include the use of hormone-responsive mammalian cells and recombinant yeast cells in cell proliferation and gene expression studies (*38,40–42*). *In vivo* quantitative gene expression assays that use PCR are being developed to investigate tissue- and response-specific effects of endocrine disruptors. Moreover, recombinant DNA technology is also being used to construct transgenic rodents that express SHBG following parturition and therefore more accurately model pharmacodynamic interactions that may occur in humans. Researchers are also investigating the possibility of combining *in vitro* and *in vivo* assays such as the development of an *in vivo* cell proliferation model. This assay would involve the use of hormone responsive cells and athymic mice to assess the ability of an endocrine disruptor to mimic sex steroid activity in inducing cell proliferation under *in vivo* conditions. Although these developments will improve the assessment of endocrine disruptors, additional research is necessary as to how endocrine disruptors impact other endocrine end points such as peptide hormone secretion, transport, metabolism, and activity.

Interactions of Organ Systems

Initial interaction of a chemical with the body occurs via inhalation, ingestion, or dermal contact. As a consequence, the primary organs involved are the lung, skin, and gastrointestinal tract. Toxicants may cause an effect at these initial sites of contact, or at distant organs because of transport, metabolism, or mediators released from these sites, as well as from the differential sensitivity of various organs. Moreover, even though initial interaction may be at one site, effects may be evident at multiple sites because of interactions between various tissues, for example, by hormones, cytokines, antibodies, neural innervation, toxic metabolites, and plasma regulatory proteins. Prominent examples of these interactive systems include the immune system, nervous system, and endocrine system, each of which is composed of numerous cell types and cell processes allowing interaction with multiple systems and resulting in homeostasis. In these cases, it is obvious that an assay for toxicity, whether toward a specific cell or molecular target, toward only a specific cell type or toward a specific cell factor, may not be able to predict toxicity to the entire organ system. For example, lack of effect of a toxicant on T-cell function cannot be used to indicate lack of toxicity to natural killer (NK) cell regulation of the immune system.

Some chemicals are organ specific with respect to their toxicities. For example, carbon tetrachloride is a liver toxicant, whereas trichloroethylene is a specific renal toxicant. However, even though a chemical displays apparent organ-specific toxicity, it may have secondary effects on other organs due to interactions between organ systems. For example, kidney toxicity that results in necrosis of the cortex may result in hematotoxicity manifest as erythropoietin (epo)-responsive anemia as a consequence of decreased kidney output of epo. Xenobiotic-induced antibody production (immunotoxicity) may result in kidney failure due to accumulation of antigen–antibody complexes in the renal glomeruli. Cadmium-induced renal toxicity results in production of antibodies to laminin that bind to placental tissue. This results in reduced uptake of methionine into the fetus, leading to developmental toxicity manifest as neural tube defects.

A further result of organ interactions is the occurrence of organ-specific toxicity due to a secondary effect. Chemically induced altered liver metabolism may result in decreased serum levels of estradiol that result in altered reproductive function. It is apparent that design of *in vitro* assays must consider organ system interactions. Because of such interactions, single isolated *in vitro* assays may be insufficient for adequate assay of the organ-toxic potential of chemicals. The replacement of systemic toxicology by alternative assays has received little scientific or regulatory attention.

In Vitro Organ Toxicity

Introduction

Today *in vitro* methods are used predominantly to analyze the mechanism of action of toxic agents. In a few exceptional cases, as in acute local irritancy testing, they can be used for classification and labeling of chemicals without further testing in animals. The situation is less promising in organotypic and systemic toxicity testing. Because the application and acceptance of *in vitro* alternatives in organ toxicity very much depends on the specific problem to be analyzed, established new organotypic tests and their limitations will be described separately for each of the organ systems described in the previous section. An overview of established organotypic tests is given in Table 4.

Current Applications of* in Vitro *Liver Models. *In vitro* liver models are currently used for metabolism, mechanistic, and screening studies (Table 5); a few validation studies have been performed. These are detailed by Guillouzo (*1,2,9,10*).

METABOLISM STUDIES. *In vitro* models from different species including humans are very useful and are widely used to get information on kinetics, metabolic pathways, induction/inhibition effect, and drug–drug interactions. There is a very good qualitative correlation in metabolic pathways between *in vitro* and *in vivo* data (*3*). Data on human material are critical to select the second species for toxicological studies and to predict potential effects of a new drug in humans (*9*). Different *in vitro* models can be used in parallel to get more information on metabolism (i.e., hepatocytes or slices, microsomes and recombinant CYPs) (*1–3*).

MECHANISTIC STUDIES. *In vitro* models are currently used for investigating mechanisms of toxicity, e.g., identification of a toxic metabolite or analysis of cellular lesions. They are not appropriate for identifying chemicals that will induce an immunoallergic toxicity.

SCREENING. Primary hepatocytes are used in the unscheduled DNA synthesis (UDS) test; the S9 fraction or microsomes from rat liver are used in the Ames test. Because of their low proliferative capacity, hepatocytes do not appear to be appropriate for the micronucleus test or the sister chromatid exchange test. *In vitro* liver models are also used for acute toxicity testing. However, their advantage over nonhepatic cells has not been well demonstrated.

REGULATORY. Only the Ames test and the UDS test with rat liver preparations are accepted in regulatory guidelines. National and international validation studies have been performed for acute toxicity. No positive conclusion has been drawn. Presently no new prevalidation or validation study is being run.

THE FUTURE USE OF *IN VITRO* LIVER MODELS. The following models should be developed since they hold promise:

- cocultures of hepatocytes with other nonhepatic cells
- immortalized cell lines expressing liver-specific functions
- genetically engineered cells expressing stable human phase 2 enzymes

Research to develop more sophisticated liver models should consider the following priorities:

- basic research to understand why drug-metabolizing enzymes are lost but remain inducible *in vitro*, whatever the culture conditions

Table 4. Established new organotypic tests.

Organ	Test	End point	Use	Reference method	Validation regulatory/ Acceptance	Limitations	Reference
Nervous system	Cells	NTE/AChE	S/A	OPIDN (hen)	OECD only	Only Ops	(7)
Nervous system	*In vitro* BBB	Multiple (funct/ perm)	Potential S/A	—	—	Only BBB	
Hematopoiesis	CFU-GM	Colony formation	A/R	Primate hematology	ECVAM-PS	Only neutropenia	(25)
Endocrine	Gene expression MCF7	Luciferase reporter gene	M/S	—	—	Low metabolic activity	(36)
Endocrine	Yeast gene expression	β-galactosidase	M/S		—	Low metabolic activity	(40)
Kidney	Epithelial barrier function tests	Transepithelial electrical resistance and permeability	M/S	—	Prevalidation stage	Only epithelial	(36)
Immune system	LLNA	Skin sensitization	S/R	GP maximization test	ACC/Val	Induction phase of sensitization	(43,44)
	MEST	Skin sensitization MEST	S/R	GP maximization test	ACC/Val		(44)
	SAR	Skin, respiratory sensitization	S/R	Inhalation tests	—	Dependent on quality of database	(45)
Embryo	Cells (embryo, stem)	Differentiation morphology	S/M	Segment II embryotoxicity	Undergoing validation	Measures only one phase of development	(34)

Abbreviations: M, mechanistic; S, screening; A, adjunct; R, regulatory, exposure limits; BBB, blood–brain barrier; NTE; neurotoxic estrase; AChE, acetylcholinesterase; OPIDN, organophosphate induced delayed neurotoxicity; Ops, organophosphates; funct/perm, function/permeability; ECVAM-PS, ECVAM prevalidation study; GP, guinea pig; ACC/VAL, accepted/validated; MEST, mouse ear-swelling test.

Table 5. Applications of human and animal *in vitro* liver preparations in pharmacotoxicological studies.[a]

Assay	Slices	Suspended hepatocytes	Cultured hepatocytes	Microsomes
Metabolic profile	+	+	+	–
Comparative interspecies metabolism	+	+	+	–
Kinetic studies	±	+	+	+
Drug–drug interactions	±	+	+	+
Induction studies	±[b]	–	+	–
Inhibition studies	+	+	+	+
Toxicity screening	±	+[b]	+	–[c]
Mechanistic studies	+	+[b]	+	–
UDS test	±	±	+	–

UDS, unscheduled DNA synthesis. ±, possible; +, currently used; –, not suitable. [a]Isolated organs not included because of the lack of availability of human liver. [b]Short-term study. [c]Can be used instead of the S9000 × *g* supernatant in the Ames test.

- development of *in vitro* models of immunoallergic toxicity (e.g., identification of new antigens)

When establishing *in vitro* liver models in toxicity testing, the following toxicological end points should be considered:

- acute nonspecific toxicity using nonhepatic cells
- toxicity in liver cells with at least two time points—acute and after a few days of exposure (to mimic chronic toxicity)
- toxicity on hepatocytes cocultured with nonhepatic target cells (e.g., kidney cells). Are metabolites formed by the liver toxic for other cells?

Cocultures of hepatocytes with other target cells should be developed and evaluated/validated under static culture conditions and under perfusion culture conditions.

New developments that will lead to a wider acceptance of complex liver and/or metabolising *in vitro* models include:

- new applications of recombinant human CYPs
- replacing the rat liver S9 from microsomes by genetically engineered cells expressing human CYPs in appropriate concentrations in the Ames test. It should be possible to mimic the human liver situation including genetic defects in some CYPs
- replacing hepatocytes in cocultures with nonhepatic target cells. It should be emphasized that human liver and human CYP450 is the gold standard. Human material has to be better standardized (functional activities, free of virus). However, ethical standards for using human tissue must be established and harmonized at the international level.

Kidney. Morphological end points are used to quantify nephrotoxicity, and light microscopy is widely used to assess cellular changes. The isolated perfused kidney, slices, and tubular fragments can be fixed and processed for routine histopathology or for immunochemical or histochemical investigations and quantitatively evaluated. Light microscopy is especially appropriate for monitoring changes in cultured epithelial monolayers. Growth and dynamic changes can easily be assessed by videoimaging techniques. If cells are grown on solid supports (glass or plastic) the number of domes (indicative of vectorial transport activity) may be quantified and changes in monolayer integrity can be delineated.

None of the *in vitro* systems described above has reached a level of standardization or validation acceptable to regulatory authorities. A number of test procedures applied in mechanistic studies of nephrotoxicity are outlined in Table 6.

An important parameter for evaluating nephrotoxicity of a chemical is the measurement of epithelial leakiness with

Table 6. Functional end points used in *in vitro* nephrotoxicity assays.

End points	Applicability	Comments
Cell viability		
Dye exclusion/retention	Renal cortical slices	Applicable in intact cell systems
Enzyme/ion leakage	Renal fragments	and slices (for example K^+ content)
	Isolated cells (in suspension)	Enzyme leakage provides
	Primary cell cultures	information on regiospecificity
	Cell lines	NB:MTT assay is not specific
		for mitochondrial damage
Synthesis of macromolecules		
Protein	Renal cortical slices	Check uptake of precursors
DNA/RNA	Renal fragments	
	Isolated cells	
	Primary cell cultures	
	Cell lines	
Matrix elements	May be useful in glomerular/ interstitial cell cultures	
Rate of proliferation		
(clonogenic assays)	Cell lines	Total protein measurements (usually reflect cell number)
Carrier-mediated transport		
Glucose	Systems containing proximal tubule cells	Can be quantified by uptake or transport
	Isolated tubules (transepithelial transport)	
Organic ions	Cultures on porous membranes	Uptake applicable to all transepithelial systems
		Check effects of specific inhibitors
		Check paracellular transport
		Not for routine use
Inorganic ions		Not widely used
Low molecular weight proteins		
Endocytosis of labeled proteins or carbohydrates		
Cultured cells of proximal tubular origin		Usually used radiolabel or horseradish peroxidase
		Easy *in vivo*/*in vitro* comparison
Barrier function	All epithelial systems, intact epithelial, or confluent cultures on porous membranes	Assessment of diffusion of extracellular markers, or electrophysiological measurements

MTT, methylthiazoldiphenyltetrazolium bromide.

indicator compounds, or the more sensitive end point of transepithelial electrical resistance generated by the vectorial transport of ions. Epithelial leakiness has been shown to be more sensitive than biochemical measures (enzyme leakage) for renal epithelial cell injury (*46*).

In vitro systems are well suited for studying interactions of chemicals with cellular structures. Initially the time– and dose–response relation should be explored. The next experiments should be conducted at relevant, sublethal doses. In addition, the concentrations used should be relevant to those delivered to the kidney *in vivo*. It is possible to elucidate toxic mechanisms by studying the following sequence:

- uptake (transport) of chemical or toxic metabolite
- interaction at target site within the cell (protein, DNA, lipid)
- check of cellular response in the following compartment: plasma membrane (transport, signal transduction, leakage), nucleus (gene expression, nuclease activation, mutation), cytoplasm/cytoskeleton (glutathione depletion, protein processing), lysosomes (protein degradation), and mitochondria (adenosine triphosphate [ATP] synthesis, fueling of transport work). The results obtained will indicate whether lethal damage (necrosis, apoptosis), repair, or proliferation occurs.

There are several improved assays for nephrotoxicity under development. Organotypic cultures have recently been improved by the combined use of microporous growth supports and application of the medium under continuous flow (perfusion) (*47*). The results to date show considerable improvement in the differentiated state of both primary cultures and cell lines at least with respect to morphology. The life span of primary cultures can be expanded up to several weeks without evidence of the marked changes in morphology indicative of dedifferentiation.

Neural Tissue. *In vitro* systems are amenable and very useful for mechanistic studies at the cellular and molecular level. As such, they have been used extensively in neurobiology and, to a minor extent, in neurotoxicology. As one of the problems of neurotoxicological research is the limited knowledge of mechanisms of neurotoxic damage, *in vitro* systems offer a useful way to apply biochemical, morphological, molecular biology and imaging techniques to the achievement of this goal (*18,19*).

When *in vitro* systems are considered as screening tools for detecting putative neurotoxicants, two important issues to be considered are the cell systems to be used and, most importantly, the end points to be measured (*20,27*). Suggested approaches involve the use of a battery of cell types such as a neuronal and a glial cell line, a more complicated system, and a nonnerve cell line. End points to be measured *in vitro* should include indicators of cytotoxicity, viability, as well as of neurotoxicity. Comparisons between nerve and nonnerve cells would offer indication of whether a chemical may have differential effects or display different potencies in these different cell types. However, it would still be an indication of cytotoxicity rather than neurotoxicity. Several end points for neurotoxicity have been proposed and many more could be suggested; these include enzymes, receptors, second messenger systems, ion channels, etc. Because of the complexity of the nervous system and the large number of potential targets, the choice of end points is crucial to avoid false positives and false negatives.

Hematopoietic System

The CFU-GM assay is the most frequently used progenitor assay in toxicology (*24–28*). It meets important prerequisites, including ease of use and portability between laboratories, low expense, high interlaboratory reproducibility, and the ability to assess toxicant effects on the actual target cell in humans (*25*). At this time, it is important to recognize that CFU-GM data are most meaningful when neutropenia is the likely *in vivo* toxicity (*26*). The *in vivo* end point of toxicity to be predicted with the CFU-GM assay is a reduction in the number of neutrophils in

the blood. There is a relationship between progenitor numbers and blood-cell counts *in vivo*, so it should be possible to predict the reduction in CFU-GM *in vivo*, and thereby the decrease in neutrophil counts, from the toxicant-dependent inhibition of CFU-GM *in vitro* (*35*).

Currently, none of the *in vitro* hematopoietic assays has been validated for toxicological purposes, although an European Centre for the Validation of Alternative Methods (ECVAM)-supported validation study will focus on the CFU-GM assay for predicting the level of acute exposure that causes severe neutropenia (*25*). In clinical oncology, the IC_{90} end point from the CFU-GM test is used for comparative toxicology of an investigational antineoplastic agent in humans, mice, rats, or dogs to determine if dose adjustment is required and to derive pharmacological targets for dose escalation (*26,28,48–50*). In cases of extreme differences in hematotoxicity between species, the CFU-GM assay could be used instead of hematology studies in primates. In pharmaceutics, the CFU-GM assay has been used to identify and halt the development of antiviral nucleoside analogues that would likely be myelosuppressive in humans (*27*). This assay also could be useful in guiding the development of antineoplastics that show activity against human tumor xenografts primarily because they are human-selective toxicants, rather than tumor-selective agents (*50*). When viewed as preclinical toxicology and used for early decisions in drug development, comparative CFU-GM toxicology could be a justification to cancel a compound that will likely cause unacceptable hematotoxicity clinically and thereby avoid useless animal toxicology.

In the near future, the CFU-GM assay could be a useful adjunct to test number 407 of the Organisation of Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals (28-day repeat-oral dose toxicology). The usual species is rat, but other species are allowed, and comparative *in vitro* toxicology in the CFU-GM assay might suggest an alternative species that is closer than the rat to human sensitivity. It could also find use as an adjunct test when rat histopathology or hematology (absolute neutrophil count) indicate bone marrow toxicity, to determine if humans will be more or less sensitive than the rat. As soon as an *in vitro* end point is identified that predicts the hematologic no observable adverse effect level (NOAEL), the assays of hematopoietic progenitors could prove useful for predicting the acute permissible exposure limits for human marrow of food contaminants and additives, industrial chemicals and environmental pollutants, and chronically administered medications (*15,16,18,33*). For prioritizing regulatory research on potential human hematotoxicants, it could be helpful to determine if human CFU-GM is inhibited at exposure levels that occur in the tissue of exposed individuals which do not lead to clinically detectable neutropenia.

Immune System. Most of the assays used to detect immunotoxicity are performed in conjunction with the standard 28-day rodent toxicity testing protocol of Karol (*51*). However, in the United States, mice, rather than rats, are more commonly used in immunotoxicity testing (*52,53*). Because of the complexity of the immune system, a battery of tests is necessary to assess immunosuppressive properties of chemicals. Currently, a two-tier approach is used, with each tier consisting of at least five assays (*54*). If positive results are obtained in the first tier of tests, chemicals are evaluated further in the second tier which includes an assessment of suppression of host resistance to either an infectious agent or a transplantable tumor.

Correlations between test outcomes and altered host defense have been studied to reduce the dependence on multiple assays and the use of large numbers of animals. Limited results indicate that a combination of three tests from the battery, each of which assesses functional capacity of immune system components or of the integrated immune system, affords excellent prediction of immunotoxicity (*55*). NK cell activity, important in the immune protection of the host against neoplastic growths, is assessed by an *in vitro* assay of NK cell-mediated killing of radiolabeled tumor cells. Peripheral blood cells from chemically dosed animals are the source of NK cells (*56*). A second cellular assay is based on analysis of lymphocyte surface antigens after chemical exposure (*57*). The third component of the testing triad is either assessment of delayed-type hypersensitivity, or the plaque-forming assay. Both tests assess the integrated function of various segments of the immune system that include T and B lymphocytes and macrophages (or other antigen-presenting cells) (*58*). It is anticipated that results obtained from this assay triad will be sufficient for regulatory decisions. This development would lead to a substantial reduction in the number of animals used for immunotoxicity screening.

Hypersensitivity is an adverse immunologic response, most typically occurring in the skin and lung, that causes tissue disturbance, disruption, or death (*59*). At least two exposures to the causative agent are necessary to effect a response: the first exposure primes the system to respond, the second and additional exposures elicit the reaction. Current regulatory guidelines require animal testing to assess the skin sensitization potential of chemicals (*44*).

Although the mechanisms of skin and respiratory hypersensitivity have not been fully elucidated, several steps in the initiation phase of the process are understood. For dermal sensitization the process is initiated by penetration of the agent through the epithelial barrier, followed by interaction with a carrier molecule. Simple mechanistic tests have been developed to address this phase of the process (*60*). One test, the local lymph node assay (LLNA), uses mice and examines the ability of chemicals to stimulate proliferation of lymph node cells (*43*). The test has undergone a validation trial and has been accepted by OECD for regulatory use. Another method well suited to assessment of sensitization potential of chemicals uses structure–activity relationships (SAR) (*45*). Several SAR models have been described for dermal sensitization and typically incorporate a parameter to estimate penetration of the skin (usually log P), and a reactivity parameter (dipole moment). Such models have been found to have a sensitivity and specificity approaching 90%.

SAR is also being developed to estimate the hypersensitivity of potential respiratory allergens. Preliminary validation of this methodology indicates a sensitivity of 86% and specificity of 95%. Other mechanistic methods are being applied to predict respiratory hypersensitivity. As IgE antibody has been implicated as contributing to the mechanism of some chemical respiratory allergies, assessment of the concentration of IgE in serum has been proposed as a screen for chemical respiratory allergy. The test is performed in mice and awaits validation studies with both positive and negative chemicals (*43,45*).

Other mechanistic assays used to estimate the potential of a chemical to elicit respiratory hypersensitivity include the basophil histamine release assay and cellular production of cytokines (*59,61*). Histamine is known to contribute to the airway constriction that typifies allergic airway sensitivity and cytokines are proinflammatory

mediators released from numerous immune cells. These assays are typically conducted in rodent species. Measurement of cytokines in serum or bronchial lavage and release of cytokines from bronchoalveolar cells also have been done in humans. These tests measure one end point of hypersensitivity and should be used in conjunction with other assays to better assess the allergic potential of a chemical.

Reproductive Toxicology. All of the established *in vitro* methods are currently used to study adverse effects of chemicals and physical agents on fertility and development in mammals. Special problems can be assessed on human material, e.g., transfer of chemicals into tubular and uterine fluid as well as into sperm and seminal fluid. These methods have been used as adjunct tests to the current long-term *in vivo* studies in rodents both for the safety assessment of drugs and for risk assessment of existing chemicals (*34*).

Whole embryo culture systems have been established in industry for screening purposes and mechanistic studies. Because the test is difficult to standardize, it has been used succcessfully in only a few laboratories. The whole embryo culture assay underwent several standardization and validation trials in Europe (*63*). For a given group of structurally related chemicals, the whole embryo culture assay can be used in-house for screening purposes. Limb bud cultures, brain cell aggregates, cultures of palatal shelves, embryonic lung, and many other organs both from rodents and even human tissues, and organs from aborted fetuses have been used extensively for mechanistic studies as adjunct tests to standard embryotoxicity tests in rodents (*33,34*). None of these assays has been established in industry or contract laboratories for screening purposes.

In vitro embryotoxicity tests using permanent embryonic cell lines failed to show any correlation to *in vivo* data in a validation trial conducted in the United States in 1988. Such assays are therefore not even used for in-house screening purposes in industry laboratories. The use of ES cells for *in vitro* embryotoxicity testing (*35*) is currently undergoing a validation trial sponsored by ECVAM. This assay is not yet established in industry.

The micromass assay using limb and brain cells is established in industry. It underwent some validation and can be used as an internal screening assay to classify chemicals within a given class by high or low embryotoxic potential. This has been shown by Flint (*64*) for fungicides and by Kistler for retinoids (*65*). A standardized protocol micromass assay was tested in an international validation trial, and Flint reported in 1993 that this assay correctly identified chemicals that are known to be teratogenic both in humans and the most common rodent species. These data have not been published. A validation trial of the micromass assay in the Netherlands was less successful, therefore the micromass assay has become less popular, even though it had been established in both industry and contract testing facilities.

A validation trial that examined both the whole embryo culture assay and micromass assay showed that the predictive value is considerably improved when the two assays are combined (*66*). Since the two tests require special equipment and a trained staff, they are established only in contract testing facilities. None of the *in vitro* tests established in the area of reproduction and fertility has been standardized, validated, or accepted for regulatory purposes.

Endocrine System. Currently available *in vivo* and *in vitro* methods used to identify endocrine disruptors and to assess the risk they pose to human and wildlife health are limited, lack sensitivity, and do not adequately account for all potential adverse effects. More specifically, it has been suggested that *in vitro* assays are not predictive of adverse *in vivo* effects and may provide false negative results due to their minimal metabolic ability, a factor necessary in the bioactivation of alleged endocrine disruptors. Therefore, new *in vivo* and *in vitro* methods are required that are complementary and assess the effect of endocrine disruptors on a number of different endocrine end points. *In vitro* methods, which generally have greater sensitivity, can be used to identify and prioritize substances that require further *in vivo* investigation as well as to provide information on potential mechanisms of action. The development of new *in vivo* assays are necessary to provide methods to evaluate the impact of endocrine disruptors on a number of different endocrine end points. The use of complementary *in vivo* and *in vitro* assays ensures a comprehensive assessment of the endocrine-disrupting activities of a substance or complex mixture.

USE OF ALTERNATIVE METHODS. It is suggested that a battery of *in vitro* and *in vivo* assays can be used in a tiered strategy to identify and assess the potency of alleged endocrine disruptors. *In vitro* assays could be used as an initial screen to identify endocrine-disrupting chemicals and complex mixtures. Results from these studies would prioritize chemicals that warrant further *in vivo* assessment and provide useful data for SARs that could also be developed to identify potential endocrine disruptors. The effect of alleged endocrine disruptors *in vivo* could then be investigated with attention directed to specific end points based on data obtained from *in vitro* assays. This strategy ensures that the most likely *in vivo* targets are assessed as endocrine disruption must be demonstrated in an *in vivo* model.

Future Prospects

Despite the rapid progress in cell and tissue culture techniques described in this paper, new methods provide only a limited amount of the information that is essential for the safety assessment of chemicals and that can sufficiently be obtained from testing in animals. Although significant progress has been made, in the near future *in vitro* tests will not allow evaluation of systemic and long-term effects of exposure to drugs and hazardous chemicals.

In contrast, *in vitro* methods are routinely used in mechanistic studies on the effects of toxic chemicals at the cellular and molecular level. Thus, mechanistic *in vitro* studies can be used as adjuncts to toxicity testing in animals and offer new prospects to risk assessment. The results of *in vitro* studies on human cells and tissues are essential to evaluate if toxic effects observed in animals are likely to occur in man.

Although most of the contributors to this document are experts in only one or two areas of toxicology, there was consensus that human cells, tissues, and organs should be the gold standard in organ-specific *in vitro* toxicology. All members of the group felt that research in this field should concentrate on improving culture techniques that will allow better preservation of human material. To make better use of *in vitro* organ and tissue cultures in the risk assessment process, access to high-quality human material will be essential. This may, however, raise ethical considerations that must be solved at the political level.

Recommendations

- Improve the infrastructure for alternative toxicity testing through the following actions: *a*) establish mechanisms to increase the availability, distribution, and use of human tissue and cells for

alternative toxicology testing; *b*) develop and make available relevant reference compounds for toxicity assessment in each organ system; *c*) organize and make available via the Internet complete *in vivo* toxicology data, including human data, that contain dose, end points, and toxicokinetics whenever available; and *d*) establish mechanisms to assure that biological material, reference chemicals, and data are subject to international standards for quality control and assurance.

- Identify and validate biological end points for target organ toxicity that can be reliably used in developing alternative strategies.
- Develop methodology to predict the target organ of toxicity using alternative methods.
- Develop procedures to assure early standardization of alternative tests.
- Minimize the number of *in vitro* tests by using the most predictive ones.
- Develop alternative methodology for detection of toxicity due to multiple organ interactions.
- SARs and computational models should be given a high priority for development and validation as alternative approaches toward reduction of animal testing.
- Develop, standardize, and validate coculture models involving bioactivating cells, tissues, and subcellular fractions with other target cells in static and perfused systems.
- Complete the development of the battery of transgenic cells that express the range of human CYPs and thereby serve as an alternative to the presently used S9 microsomes derived from animals in assessments predictive of human risk.
- Evaluate the origin and suitability of cultured cell lines to assess particular toxicants. For example, use tumor-derived cell lines in alternative methods only if they are derived from tumors that are not resistant to cytotoxic anticancer drugs.

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