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4.0 IN VITRO METHODS FOR ORGAN-SPECIFIC TOXICITY

4.1 Introduction

Breakout Group 3 reviewed *in vitro* methods that can be used to predict specific organ toxicity and toxicity associated with alteration of specific cellular or organ functions. The Breakout Group then developed recommendations for priority research efforts necessary to support the development of methods that can accurately assess acute target organ toxicity.

Knowledge of the effects of acute exposure to unknown materials is needed early in the development of new products and chemicals. Researchers who are using new chemicals in the laboratory need to know what types of safety precautions they need to take when handling these materials. Manufacturers must have some idea of the safe levels of exposure before they can develop the processes and build the facilities to safely manufacture the materials. The toxic doses also define precautions that must be taken when shipping materials, and govern the appropriate response of emergency personnel in case of accidental spills. Planned or inadvertent single-dose exposure of specific human or other populations may also occur, such as from accidental ingestion of common household materials, application of single use pesticides, and some pharmaceuticals.

The Breakout Group was asked to review *in vitro* methods for predicting specific target organ toxicity. Specifically the Breakout Group was asked to do the following: (a) identify the most important areas where *in vitro* methods are needed; (b) review and comment on the current status of *in vitro* methods to predict target organ toxicity; and (c) prioritize the need for future research in this area. In addition, the Breakout Group considered where it would be necessary to include prediction of specific target organ toxicity in developing an *in vitro* program to replace the current acute oral toxicity assays used in hazard classification systems.

The scope of the remit was very broad and the Breakout Group proceeded by identifying the

organ systems where failure could lead to lethality after acute exposure. The Breakout Group reviewed each system individually, and then proposed a scheme for including the important endpoints identified into a replacement test battery for acute toxicity.

4.1.1 Regulation of Industrial Chemicals and Pesticides

A representative (Dr. Karen Hamernik) of the U.S. EPA related the needs of an agency that regulates industrial/commodity chemicals and pesticides. In addition to their use in assigning an international hazard classification, the results of acute toxicity tests are used to set doses for *in vivo* cytogenetics assays, acute neurotoxicity tests, and, occasionally, for other types of rodent tests. Dose setting may utilize LD50 information and dose response data over a range of doses for a given test material. In addition, information on the effect of single exposures is gathered during acute neurotoxicity tests, developmental toxicity tests, and metabolism studies. In these tests, multiple endpoints may be measured and the results can be used for hazard and risk assessments for single-exposure scenarios.

The U.S. EPA is concerned with organ-specific effects -- including their severity, onset, and duration -- that become apparent from various test material exposure scenarios including acute, sub-chronic, or chronic exposure. Some study protocols provide reversibility-of-effects information. Information on organ-specific effects may have an impact, at least in part, on risk assessment methods depending on the effect of concern, whether a mechanism for toxicity can be proposed or identified, and on the available dose-response information. For instance, organ-specific effects may impact decisions on whether to regulate based on cancer or non-cancer endpoints, to use linear or non-linear models, and whether to use dose-response data or benchmark dose approaches.

How organ-specific effects impact risk assessment depends to some extent on where the effects occur on the dose-response curve, what types of effects are seen and their severity, and the nature of the exposure. Examples include the

presence of clear toxic effects such as necrosis and changes in enzyme activities or elevations in hormone levels that may be considered precursors to possible longer-term toxic, or even carcinogenic, effects. The impact of these effects may depend upon whether they are seen only in adult animals, young or adolescent animals, or during *in utero* exposure. Toxicity data are used for human risk assessment and to provide clues for potential concerns for effects in wildlife.

In the United States, organ-specific effects seen in toxicity studies may trigger Food Quality Protection Act-related issues such as the possibility of grouping chemicals with common modes of action or mechanisms for cumulative risk assessment. Certain organ-specific effects may serve as a starting point to look at questions related to human relevance. The presence of such findings may trigger the need for additional studies to support the suspected toxicological mechanism.

4.1.2 Regulation of Pharmaceuticals

A representative (Dr. David Lester) of FDA/CEDR related the needs of an agency that regulates pharmaceutical materials. CEDR does not ask for, nor regulate, non-clinical toxicity testing, and does not use estimates of the LD50 value in its assessments. In general, the agency does not find identification of specific organ toxicity after single-dose acute exposure useful since most pharmaceuticals are given as multiple doses.

The results of acute toxicity tests are not useful in establishing dosing regimes because most pharmaceuticals are developed for multiple use. Acute effects are more important for oncologic drugs because the margins of safety may be smaller. Single-dose studies may also be useful for developing imaging agents where it is important to understand tissue distribution after a single exposure.

In vitro studies are often performed in drug development as part of the effort to understand the disease process or to understand the actions of the drugs on specific cells. In drug development, the risk assessments are based on the total dose of the material given and not on the tissue

concentration. *In vitro* studies have been used in setting doses for initial human exposure to cancer therapeutics, but otherwise are rarely used for dose setting because current methods cannot extrapolate from the *in vitro* concentration to the dose that must be given to achieve similar effects *in vivo*. Animal studies may be used for initial dose setting for early clinical studies, but these are usually not acute, single-exposure studies.

4.1.3 U.S. National Toxicology Program (NTP)

The Breakout Group also heard a presentation (from Dr. Rajendra Chhabra) on the use of acute oral toxicity data by the National Toxicology Program (NTP). The NTP does not find it necessary to use acute studies to set doses for subchronic studies; instead, researchers go directly to 14- or 90-day studies. If there are sufficient data on the chemical of interest, then they are often able to avoid a 14-day study. The results of 90-day studies in rodents are used to set doses for chronic studies and also to determine what specific types of additional studies may be needed (i.e., reproductive, cancer, neurotoxicology, etc.). To facilitate decision making and reduction of animal use, the NTP adds several endpoints to the 90-day study including sperm morphology, immunotoxicology, neurotoxicology, and a micronucleus test.

The NTP is evaluating a battery of *in vitro* tests that might reduce the need for 14-day dermal toxicity studies. The tests include:

- The bovine corneal opacity test;
- The skin permeability assays;
- The EpiDerm™ model for dermal irritation/corrosivity;
- A neutral red uptake (NRU) assay for systemic toxicity;
- A primary rat hepatocyte assay for hepatic toxicity.

Five chemicals have been tested in this battery. The 14-day *in vivo* rodent study costs about \$150,000, uses 120 animals, and takes about six months to perform. An accurate battery of *in vitro* tests would be less expensive in both time and cost.

4.1.4 Initial Considerations

The Breakout Group agreed for the purposes of this exercise to define acute toxicity as “*toxicity occurring within 14 days of a single exposure or multiple exposures within 24 hours*”. For evaluating chemicals for acute toxicity, the Breakout Group identified the following major organ systems as the ones that need to be considered:

- Liver;
- Central nervous system;
- Kidney;
- Heart;
- Hematopoietic system;
- Lung.

Damage significant enough to cause death can occur to these systems after a single acute exposure. The Breakout Group recognized that local effects of xenobiotics on the skin, gastrointestinal tract, and eye may also be important, but agreed to focus on systemic effects rather than local effects. The Breakout Group also recognized that the developing embryo may suffer serious, even lethal, consequences after a single acute exposure to a xenobiotic. However, the Breakout Group felt these effects are adequately evaluated by the standard battery of tests for reproductive and developmental effects and do not need to be included as part of an *in vitro* battery to replace the acute toxicity tests.

The Breakout Group discussed the use of rodent cell cultures as the basis of *in vitro* tests to predict acute toxicity. The work of Ekwall (Ekwall et al., 2000) indicates that for general cytotoxicity cells of human origin correlate best with human acute lethal blood concentrations. There are well recognized species differences in response to many classes of xenobiotics that must be taken into account as systems are developed to predict effects specific to individual organ systems. Considering the species differences currently recognized and other differences that might not yet be identified, the Breakout Group recommends that every effort should be made to use human-derived cells and tissues, preferably normal, as the basis for *in vitro* assays since data from the *in vitro* studies will ultimately be used to predict toxicity in humans.

4.2 Review of a Proposed Screen to Elucidate Mechanism of Injury

The Breakout Group examined specific endpoints or organ systems. Both *in vivo* and *in vitro* systems are used extensively in industry and academia to aid in the understanding and prediction of mechanisms of toxicity. The review attempted to highlight situations where *in vitro* studies provide information at least as useful and often more useful than *in vivo* studies and to identify areas where further research is needed before *in vitro* techniques will be able to replace whole animal studies.

The Breakout Group first reviewed a program using eight different normal, human epithelial cell lines or primary cells for initial toxicity screening to elucidate mechanisms of injury by measuring comparative tissue-specific cytotoxicity of cancer preventive agents (Elmore, 2000; Elmore, in press). Tissue-specific cytotoxicity was assessed using cell proliferation at three days and five days, mitochondrial function, and PCNA or albumin synthesis (hepatocytes only) as endpoints. The cells used were early passage cell lines following cryopreservation or were primary cultures (hepatocytes) and included liver, skin, prostate, renal, bronchial, oral mucosa, cervix, and mammary tissues.

The results suggest that different chemicals induced unique tissue-specific patterns of toxicity. Changes in toxicity following three and five day exposures provide additional information on both delayed toxicity and the potential for recovery. Confirmation of the predictive trends was confirmed with several agents in keratinocytes using 14-day cultures with multiple exposures. Ongoing studies will compare the *in vitro* data with blood levels from preclinical animal studies, and plasma levels and observed side effects from clinical trials.

4.3 In Vitro Methods for Determination of Acute Liver Toxicity

Adequate liver function is critical to the survival of an organism. The liver is at high risk for injury because it is actively involved in metabolizing xenobiotics, and because the liver is exposed first

to materials absorbed from the gastrointestinal tract. The liver also excretes many materials via the bile and this puts the biliary system at risk for toxicity as well. For these reasons, one of the highest priority needs is for a test system that can accurately evaluate the effects of xenobiotics on the liver. Test systems need to be able to assess both the potential for hepatic toxicity and whether the liver will be able to metabolize the test chemical either to a more or less toxic moiety. Xenobiotics may also affect the biliary tract, and an *in vitro* system to investigate these effects will also be needed.

4.3.1 Available Non-Animal Models

Available non-animal models include metabolically competent animal or human liver cells. Such cells have been cryopreserved and cryopreserved human cells are available commercially. The cells of human origin have a short life span, but they can be obtained with certain well-characterized metabolic profiles including specific active P450 systems. Immortalized human cell lines, some of which have been transfected to express specific recombinant phase I or II enzymes are also available, but most cell lines are limited to expressing only one enzyme.

Assessment of the potential for hepatic metabolism is possible using isolated hepatocytes (Cross and Bayliss, 2000; Guillouzo, 1997) and cell lines. Liver microsomes are used in high throughput screening assay systems to determine the extent of metabolism of a parent compound. Whole liver homogenates, subcellular fractions, and liver slices are also commonly used in basic research on hepatic function and toxicology (Guillouzo, 1998; Parrish, et al., 1995; Ulrich et al., 1995; Waring and Ulrich, 2000). A report on the ECVAM Workshop on the Use of Tissue Slices for Pharmacotoxicology Studies includes a comprehensive review of the use of liver slices in toxicology (Bach et al., 1996). These systems can be robust, but the supply of human liver tissue is limited and is decreasing as more donor liver is being used for transplantation.

Recently, more complex systems have been developed in an attempt to better mimic hepatic

function. Cell culture techniques that involve sandwiching liver cells between layers of collagen can be used to study induction of metabolic function, but it is difficult to examine the hepatocytes after treatment because of the collagen in the system. Liver cells can also be cultured as small compact spheres of cells. As these spheroids grow, they tend to become necrotic in the center so their usefulness in toxicology needs to be established.

There have been some attempts to develop *in vitro* systems to study effects on biliary function. A couplet system made up of two hepatocytes with bile canaliculi attached has been described. This system is very labor intensive and currently would not be viable as a routine test system but is useful as a way to study mechanisms of cholestasis. In addition, liver fibroblasts can be cultured for the study of mechanism of hepatic cirrhosis.

4.3.2 Specific Endpoint Measurements

As *in vitro* systems for hepatic function are developed to replace animals in acute toxicity studies, the specific endpoints which should be considered are changes in enzyme systems, membrane damage, changes in mitochondrial function, changes in albumin synthesis, and possibly cell detachment. It will be important to identify systems that express the most important metabolic systems present in normal human liver. The Breakout Group discussed the need for multiple cell lines to represent the known diversity of enzyme systems expressed by the human population. While such systems are very useful in drug development, the Breakout Group recognized that this degree of sophistication is not available with the current *in vivo* systems and should not be required for a replacement system for acute toxicity.

4.3.3 Future Needs

Future work in the area of hepatic toxicology will depend upon the development of more robust models that are as metabolically competent as mature human hepatocytes *in vivo*. Pharmaceutical companies are currently using *in vitro* assays of hepatic function for screening new drugs and as their methods become more readily

available, they may be useful in acute toxicity testing. An ILSI HESI Genomics Subcommittee is assessing changes in gene expression that occur in response to several prototypic chemicals, including hepatotoxicants, and will be attempting to correlate the gene expression changes with changes in various biological and toxicological parameters.

Two methodological issues need to be addressed as *in vitro* methods are developed and evaluated. First, when culturing liver cells, it is vital that the cells are constantly monitored to ensure they are still expressing the desired characteristics and this monitoring must be built into protocols. Second, there is considerable variability in enzyme function between cells from different individual donors, and for toxicity testing it will be necessary to agree upon the cell characteristics needed for an appropriate test system that will best represent the overall human population.

There is a high-priority need to develop a system for regulatory use that will be able to recognize which compounds the liver will metabolize to another compound or compounds. To replace whole animal, systems must be devised that can also determine the effect of the product or products of hepatic metabolism on other organ systems in a dose responsive manner.

There is a need for a worldwide database comparing human *in vitro* and *in vivo* data for hepatic toxicity. Scientists attempting to develop hepatic systems for toxicity testing are encouraged to share methodology and cell lines. Collaboration among laboratories would increase the pace of research and avoid development of multiple and competing test methods.

4.4 *In Vitro* Methods for the Determination of Acute Central Nervous System (CNS) Toxicity

Neurotoxic effects after a single dose are often expressed as either overall CNS depression resulting in sedation, or excitation, generating seizures or convulsions. The molecular mechanisms for these states may be related to very specific toxicant-target interaction, or the targets may be general for all cell types but are involved in critical functions in neurons. Because

CNS effects can lead to acute lethality, a neurotoxicological screen should be performed when certain criteria in the tiered test battery, as described in Section 4.10.1, have been fulfilled. Briefly, the steps are physico-chemical or other information indicating that the toxicant can pass the BBB, low basal cytotoxicity (high EC₂₀ or EC₅₀ values) in non-neuronal cells, low hepatotoxicity, and no evidence of impaired energy metabolism at non-cytotoxic conditions. If these initial criteria are fulfilled, investigations of the neurotoxic potential of the test material must be carried out. The cellular targets can be either general or very specific functions.

4.4.1 *Important General Cellular Functions for CNS Toxicity*

Examples of important general cellular functions that upon impairment may cause severe brain damage after acute exposure are decreases in resting cell membrane potential, increases in intracellular free calcium concentration ($[Ca^{2+}]_i$), and formation of free radicals and reactive oxygen species (ROS). Cytotoxicity may, eventually, occur as a result of severe insult to these cellular functions. In some cases, astrocytes are the immediate target and the toxic reaction may appear as astrocyte activation and formation of neurotoxic cytokines. An early marker for acute astrocyte activation is increased glial fibrillary acidic protein (GFAP) expression.

4.4.1.1 *General Endpoints*

Endpoints that can be assessed include cell membrane potential, increased $[Ca^{2+}]_i$, and free radical formation that can easily be measured by fluorescent probes or by simple spectrophotometry. Cytokines and GFAP levels can be determined by immunochemical techniques, such as ELISA, or by mRNA quantification (e.g., *in situ* hybridization, RT-PCR, or gene array analysis). Most assays can be performed on adherent cells in microtitre plates, which make them useful for high throughput screening.

4.4.1.2 *Cell Models for General Functions*

Several cell models are available. General cell functions can be studied in cell types that possess a near normal cell membrane potential and

aerobic energy metabolism. Certain differentiated human neuroblastoma cell lines, such as SH-SY5Y, fulfill these criteria and are easy to obtain, culture, and differentiate. Human brain neural progenitor cell lines (e.g., NHNP and NT2) are now widely available. The NHNP cell line has the advantage that in culture it differentiates into a mixture of neurons and glia. It can be passed through numerous passages and forms spheroids in suspension (Svendsen et al., 1997). Glial cell lines are generally poorly differentiated even though there are reports of some GFAP-expressing human cell lines (Izumi et al., 1994; Matsumura and Kawamoto, 1994). Rat glioma 9L cells have been reported to manifest astrogliosis upon chemical exposure (Malhotra et al., 1997). Nevertheless, primary rat astrocyte cultures are used in most studies on astrocyte activation.

4.4.2 Important Specific Functions for CNS Toxicity

Specific functions can be measured by assessing neuronal targets that will cause acute CNS depression or excitation if their functions are impaired. These functions are voltage operated Na^+ , K^+ , and Ca^{2+} channels and the ionotropic glutamate NMDA, GABA_A , and nicotinergic acetylcholine (nACh) receptors. Furthermore, severe intoxication may occur after acute exposure to cholinesterase inhibitors. Besides the acute effect on cholinesterase function, delayed neuropathy may also be evident after a single dose.

4.4.2.1 Specific Endpoints

Ion fluxes over the cellular membrane can be estimated by using various ion-selective fluorescent probes. However, upon stimulation, effects on ion channels or receptors change the net membrane potential. Eventually, this will result in altered Ca^{2+} -fluxes and $[\text{Ca}^{2+}]_i$, which in turn will affect transmitter release. Therefore, effects of toxicants on receptor and ion channel functions may be detected as increased or decreased $[\text{Ca}^{2+}]_i$ (Forsby et al., 1995) or neurotransmitter release (Andres et al., 1997; Nakamura et al., 2000; Smith and Hainsworth, 1998; Wade et al., 1998). The effects may be evident directly by the toxicant itself, but also after applied stimuli such as potassium-evoked

cell membrane depolarization, possibly in the presence of receptor agonists. Acetylcholine esterase (AChE) activity in neuronal cells can be measured in differentiated cells such as SH-SY5Y cells. Evaluating changes in the ratio between AChE and neuropathy target esterase (NTE) has been proposed as a method for estimating the risk for delayed neuropathy (Ehrich et al., 1997).

4.4.2.2 Cell Models for Specific CNS Functions

Cell models for studies on specific CNS functions should be of human origin, mainly because certain enzyme structures and receptor sub-unit expressions differ among different species. Furthermore, the level of cellular differentiation is crucial. The cell lines must, in most cases, be treated with differentiating agents such as retinoic acid to express features of normal, adult neurons. Cells that are transfected with genes expressing specific receptor and ion channel proteins can also be useful for studies on specific functions.

One example of non-primary neuronal cells is the human neuronal progenitor NT2 cells derived from a teratocarcinoma. The NT2 cells can be terminally differentiated to NT2-N cells after treatment with retinoic acid and mitosis-arresting agents after months in culture. NT2-N cells express functional NMDA and GABA_A receptors (Younkin et al., 1993; Munir et al., 1996; Neelands et al., 1998). The previously cited NHNP neural human brain progenitor cell line could also serve as an important model system for neurotoxicity screening (Svendsen et al., 1997). It is not as well characterized as the NT2 line but deserves investigation. Alternatives to NT2-N may be native or differentiated human neuroblastoma cell lines (e.g., SH-SY5Y, IMR32 and CPH100). However, their receptor sub-unit expression and receptor function may vary from normal receptors present in adult brain tissue.

Co-cultures of neuronal and glial cells may be used for studies on interactions between neurons and glia cells. For instance, NT2 cells differentiate and establish functional synapses when they are cultured on astrocytes (Hartely et al., 1999). Upon differentiation, the NHNP cell line cultures contain a mixture of astrocytes and neurons varying in ratio from 1:9 to 2:3. In

suspension, the NHNP cells form spheroids (see Clonetics web site). Reaggregated embryonic brain cultures have been recommended for screening of neurotoxic compounds (Atterwill, 1994) but significant further work on this promising model is needed before it can be used as a standard test method.

4.4.3 Future Needs

Some endpoints, assays, and cell models for the more general endpoints have been studied and used extensively, which make them ready for formal validation. However, most assays and cell models determining effects on special functions still need significant basic research before they will be useful in screening systems.

4.5 In Vitro Methods to Assess Blood-Brain Barrier (BBB) Function

The CNS is dependent on a very stable internal environment. The BBB helps maintain this stable environment by regulating all uptake into and release from the brain of substances involved in CNS metabolism. The barrier acts as a functional interface between the blood and the brain, rather than as a true barrier, and this function is localized to the brain capillary endothelial cells. These cells differ from endothelial cells in other organs in that they form tight junctions. They have a higher turnover of energy and thus contain numerous mitochondria; they have a low endocytotic activity. Furthermore, they express specific transport proteins and enzymes. Water, gases, and lipid-soluble substances may pass the BBB by simple diffusion whereas glucose, monocarboxylic acids, neutral and basic amino acids, and choline are taken up from the blood by active processes. Ions pass the BBB very slowly and proteins generally not at all. Weak organic acids, halides, and potassium ions are actively transported out of the CNS.

From a toxicological viewpoint, three aspects of the BBB are of interest: (a) the BBB regulates uptake and release of endogenous substances and also xenobiotics, (b) toxic substances may interfere with the structural and functional properties of the BBB, and (c) certain parts of the CNS (e.g., areas in the hypothalamus and the choroid plexa), have poorly developed BBB

functions. The latter is also true for all parts of the embryonic and juvenile brains.

Several authors and working parties have identified the need for a reliable *in vitro* model of BBB functions as being essential for the development of alternative methods for use in tests of acute systemic toxicity, neurotoxicity, and in drug development (Balls and Walum, 1999; Ekwall et al., 1999; Janigro et al., 1999; the ECVAM workshop on *In Vitro* Neurotoxicity [Atterwill et al., 1994], the ECVAM Neurotoxicity Task Force, [1996, unpublished], and the BTS Working Party Report on *In Vitro* Toxicology, [Combes and Earl, 1999]). ECVAM is currently supporting a prevalidation study of *in vitro* models for the BBB. The study largely follows the recommendations published by Garberg (1998).

4.5.1 Endpoints for Acute Toxic Effects

For acute toxic effects, there are two endpoints for toxic insult to the blood brain barrier: (a) partial or complete breakdown of the barrier function (i.e., effects on the ability of the BBB to exclude endogenous and exogenous substances) and (b) changes in the specific transport capacity of the BBB. There is a need to measure the ability of the normal BBB to transport toxicants into or out of the brain.

4.5.2 Models

Models currently being assessed in the ECVAM-sponsored prevalidation study include:

- Immortalized endothelial cell lines of both human and animal origin;
- Primary bovine endothelial cells co-cultured with glial cells;
- Barrier-forming continuous cell lines of non-endothelial origin.

Preliminary results from the ECVAM prevalidation study, as well as previously published results, show that the rate of penetration of compounds that pass the BBB by simple diffusion can be estimated by the determination of log P, or by the use of any cell system that forms a barrier (e.g., MDCK or

CaCo2 cells). This means that the distribution of lipophilic compounds over the BBB can be determined simply, and that the first aspect of acute toxic effects (i.e., impairment of the barrier function [see above]) can be studied in continuous cell lines, provided they are able to form tight junctions.

With respect to the second endpoint, impairment of the transporter functions and the transport-mediated brain uptake, the situation is different. The modeling of these features of the BBB ideally requires an *in vitro* system with a high degree of differentiation, including the significant expression of all transporter proteins representing species-specific properties. At present, this can only be achieved in primary cultures of brain endothelial cells co-cultured with brain glial cells.

A model presented by Stanness et al. (1997) shows development of a dynamic, tri-dimensional *in vitro* culture system (DIV-BBB) that mimics the *in vivo* BBB phenotype more closely than other models in use. In this system, cerebral endothelial cells are cultured in the presence of astrocytes using a hollow fiber technique. The fiber cartridge, representing artificial capillaries, is exposed to a luminal pulsatile flow of medium. Although a very good model for the *in vivo* situation, the DIV-BBB model may be too resource intensive to be of practical use in a screening situation.

4.6 *In Vitro* Systems to Study Kidney Toxicity

The major effect seen in the kidney after acute exposure to a nephrotoxin is acute tubular necrosis. In approximately 90% of the cases, the changes are seen in the proximal tubular cells (proximal to the convoluted tubules). These cells have high metabolic activity and a significant concentrating function, both of which put them at increased risk for damage. There are a much smaller number of substances that are toxic to the distal tubular cells. While acute toxicity in tubular cells is highly significant and can be fatal, it is important to recognize that these cells have great regenerative capacity and with adequate treatment and time will repopulate and replace the destroyed cells.

There are a few substances that cause direct glomerular damage which is more serious because glomerular damage is permanent resulting in the loss of the affected nephron. Although the kidney has a considerable reserve capacity of nephrons, it is important to understand the effects of a reduction of this reserve capacity particularly in individuals, such as the elderly, who may already have a reduced number of nephrons.

A comprehensive review of the use of *in vitro* systems to assess nephrotoxicity has been completed by ECVAM and was used as the basis for the discussion (Hawksworth et al., 1995). *In vitro* systems will need to utilize metabolically competent kidney tubular cells. This should not be as difficult as liver systems since much is known about the metabolic function of renal tubular cells, and there does not appear to be significant variability between individuals. In addition to direct cytotoxicity, *in vitro* systems must be able to evaluate the barrier function of the kidney. A system to assess this parameter is currently being studied in Europe, with support from ECVAM. In addition, *in vitro* systems may need to assess transport functions. At this time it is not clear how important these functions are in acute toxicity. It is also not known how much variability exists in these functions from one individual to another. The specific transport functions are not completely characterized and more basic research is needed before test systems can be developed.

It is possible to measure kidney function in a non-invasive fashion in humans who are exposed to low levels of xenobiotics, for instance, in occupational exposures. It would be valuable to evaluate the correlation of the results from *in vitro* toxicity tests with information from humans.

4.7 *In Vitro* Methods to Assess Cardiotoxicity

Cardiovascular toxicity can result from excessive accumulation of toxic chemicals within the tissue, cardiovascular-specific bioactivation of prototoxins, and/or chemical interference with specialized cellular functions. Because a cardiotoxic insult interferes with the ability of the

heart to pump blood through the vasculature, blood flow to major organs is often compromised. Vascular toxicities are often characterized by slow onsets and long latency periods and are not usually important in acute toxicity; however, changes in arterial pressure and blood flow control may be significant in acute effects.

The pathogenesis of cardiovascular injury often involves the elucidation of oxidative mechanisms and many cardiovascular disorders are characterized by loss of redox homeostasis. The central role for oxidant mechanisms is consistent with studies which show evidence of beneficial effects of antioxidants provided to patients with coronary heart disease (Napoli, 1997). The vascular production of reactive oxygen metabolites increases substantially in disease states (Harrison, 1997). Links between cardiovascular and cerebro-vascular disorders have also been established. During periods of emotional stress, adrenaline toxicity to vascular endothelial cells may involve its deamination by monoamine oxidase A to form methylamine, a product further deaminated by semicarbazide-sensitive amine oxidase to formaldehyde, hydrogen peroxide, and ammonia (Yu et al., 1997).

4.7.1 *Perfused Organ Preparations*

Perfused organ preparations are currently the most representative of the *in vivo* situation. Aortic preparations are most preferred; they can be readily excised, perfused, and super-perfused with appropriate buffers, (Crass et al., 1988). Perfused preparations are advantageous because they retain the level of structural organization found *in vivo*. Toxin-induced changes in physiologic/pharmacologic sensitivity and changes in excitability and/or contractility can be readily evaluated. The biological actions of nitric oxide, a soluble gas synthesized by the endothelium, was first discovered using perfused preparations. Because perfused organ preparations require harvesting fresh tissue, better methods are still needed. In addition, significant limitations of perfused preparations in toxicity testing include the small number of replicates that can be processed, the time required for isolation, and the provision that the system can only be used

for short periods of time because of rapid loss of viability. Parameters measured include: (a) time to peak tension, (b) maximal rate of tension development, and (c) tension development. Oxygen concentration of the perfusate provides an index of myocardial oxygen consumption. Pin electrodes can be used to obtain electrocardiographic readings. Measurements of contractility and stress development can be used to evaluate effects of drugs and chemicals.

4.7.2 *Isolated Muscle Preparations*

Isolated muscle preparations consisting of strips of atrial, ventricular or papillary muscles (Foex, 1988), or segments from vascular beds (Hester and Ramos, 1991) can be super-perfused with oxygenated physiologic solutions for measurements of tension development. The pre-load and after-load placed on the tissue can be controlled accurately to evaluate isometric force development, isotonic force development, and quick-release contractions. Oxygenation of the tissue is a function of diffusion, and the thickness of the strips and oxygen concentration in the solution bath must be carefully monitored. The stability of these muscle strips is limited to short time periods. Because many preparations can be made from each animal, these systems use less numbers of animals than perfused organ preparations.

Isolated preparations have been used to examine the angiotoxic effects of ethanol (Rhee et al., 1995), acetaldehyde (Brown and Savage, 1996), palytoxin (Taylor et al., 1995), and cadmium (Ozdem and Ogutman, 1997). Regional differences in physiologic and pharmacologic responsiveness must be considered in developing strategies that examine vasculotoxic responses. Aortic rings exhibit higher sensitivity to norepinephrine than mesenteric artery rings, while the reverse effects are found with serotonin. However, no differences in sensitivity to KCl and CaCl₂ were observed (Adegunloye and Sofola, 1997). Differences between the two vessels appear dependent on agonist ability to mobilize calcium from intracellular stores.

4.7.3 Organ Culture Preparations

Organ culture preparations offer long-term stability as compared to other *in vitro* preparations. Whole fetal hearts from mice and chicks have allowed the study of processes associated with myocardial cell injury (Ingwall et al., 1975; Speralakis and Shigenobu, 1974). Organ-cultured blood vessels have led to elucidation of structural/functional relationships of the vessel wall matrix (Koo and Gottlieb, 1992). However, organ culture of rat aortic rings results in significant loss of contractile responsiveness to different agonists within 24 hour (Wang et al., 1997).

4.7.4 Tissue Slice Preparations

Tissue slice preparations of cardiac tissue have been characterized as models to evaluate toxicity of xenobiotics (Gandolfi et al., 1995) and could be useful in toxicity testing applications (Parrish et al., 1995).

4.7.5 Single-Cell Suspensions

Single-cell suspensions of embryonic or neonatal cells that are derived from ventricular, atrial, or whole heart tissue can be easily prepared by enzymatic and/or mechanical dissociation of the tissues. Adult hearts can also be dissociated by a modified recirculating Langerdorff perfusion that yields a large proportion of cells which remain rod shaped and are quiescent in medium containing physiologic calcium levels (Piper et al., 1982). The anatomic distribution of cells within the walls of large and medium-sized mammalian vessels facilitates the isolation of relatively pure suspensions of fibroblastic, endothelial, or smooth muscle cells. In contrast to cardiac preparations, vascular cells from embryonic, neonatal, and adult vessels can be efficiently isolated in calcium- and magnesium-containing solutions.

Myocardial cell suspensions represent a heterogeneous population of muscle and non-muscle cells. Neonatal myocytes are remarkably resistant to injury and exhibit variable degrees of beating shortly after isolation. In contrast, spontaneous beating of adult cardiac myocytes is thought to be due to uncontrolled leakage of calcium through a permeable plasma membrane.

Adult cardiac myocytes are mechanically at rest when properly isolated suggesting that functional differences in regulation exist between adult and neonatal cells. Isolated cells can be microinjected with fluorescent dyes for the assessment of multiple cellular functions following exposure to toxic chemicals. The viability of cells in suspension decreases rapidly as a function of time. Investigators rarely use these cell suspensions for more than four hours.

Changes in cell function or contractility can be assessed using these models. Because heart failure, in some instances, is characterized by contractile dysfunction of the myocardium and elevated sympathetic activity, cell function or contractility is of concern (Satoh et al., 2000). It has been demonstrated that adult rat ventricular myocytes in culture show signs of decreased contractility when exposed to adrenergic stimulation by norepinephrine + propanolol for 48 hours. This result seemed to be due to decreased Ca (2+)-ATPase. Consequently, sympathomimetic agents or other chemicals that decrease Ca (2+)-ATPase would have similar activity.

A number of anthracycline antineoplastic agents are known to cause cardiac cytotoxicity that can be severe and often irreversible. Doxorubicin and 4'-epirubicin significantly depress myocyte contractility in isolated neonatal and adult rat ventricular myocytes (Chan et al., 1996) but the etiology of the toxicity has not been determined definitively (Sawyer et al., 1999). The effect can be assessed by visualizing the beating of the myocytes (Jahangiri et al., 2000) or by measuring calcium flux using fluorescent dyes (Trollinger et al., 2000). Cultured fetal chick cardiac myocytes have also been used to study the toxicity of hydrogen peroxide and certain agents which can protect against such toxicity (Horwitz et al., 1996).

4.7.6 Models Using Cell Lines

Cardiac cell lines are generally preferred for the evaluation of chemical toxicity following prolonged exposures or following multiple challenges *in vitro*. Primary cultures can be established with relative ease from cell

suspensions of cardiac and vascular tissue. However, they must be characterized at the morphologic, ultrastructural, biochemical, and functional levels before being used in cytotoxicity testing applications because they undergo variable degrees of dedifferentiation, including loss of defined features and cell-specific functions. Vascular endothelial and smooth muscle cultures can also be established using explant methods, but the explant method selects cells with a growth advantage. Neonatal and embryonic cells of cardiac origin proliferate readily under appropriate *in vitro* conditions. Although adult cardiac myocytes do not divide in culture, the ability of cardiac myocytes to divide is only repressed and not completely lost (Barnes, 1988). A human fetal cardiac myocyte cell line was developed by transfection with the SV40 large T antigen to stimulate myocardial cell division, and many of the morphologic and functional features of human fetal cardiac myocytes were preserved (Wang et al., 1991).

4.7.7 Endpoints That Can Be Assessed In Vitro

Flow cytometry and computerized evaluation of cell images have added to toxicity evaluations of cardiac myocytes. Toxicity can also be evaluated based on the arrhythmogenic potential of chemicals (Aszalos et al., 1984). Ionic homeostasis can be used as an index of disturbances in the structural and functional integrity of the plasma membrane. Use of co-cultures of myocytes and endothelial cells or smooth muscle cells in the progression of the toxic response emphasizes the importance of cell-cell interactions (Saunders and D'Amore, 1992).

4.7.8 Future Research Needs

Vasculitis may need to be assessed by *in vitro* methods. It can be present in numerous forms such as lymphocytic vasculitis and leukocytoclastic vasculitis, the latter usually affecting the skin (Gupta et al., 2000). The most common type of vasculitis is Giant cell arteritis (Gonzalez-Gay et al., 2000), which generally involves large and medium-sized blood vessels. Further work will be needed to identify *in vitro* systems to assess this endpoint.

Certain drugs have the potential to alter the QT interval in the heart, producing ventricular arrhythmias and it will be necessary to develop systems to detect this effect. Halofantrine, an antimalarial drug, has been reported to produce such effects, and some drugs have been implicated in the sudden death of patients from ventricular arrhythmias (Champeroux et al., 2000). In a review by Champeroux (2000), different methodologies have been investigated as possible ways of examining this potential -- *in vitro* as well as *in vivo*. These include isolated cardiac tissues, Purkinje fibers, or papillary muscles. Wesche (2000) also used an isolated perfused heart model and isolated ventricular myocytes to determine potential cardiotoxicity associated with antimalarial drugs (Wesche et al., 2000).

A final important effect of acute exposure to xenobiotics is aseptic shock, which is associated with a fall in blood pressure. This is a systemic effect and no method of measuring or modeling this effect *in vitro* could be identified at this time. Further work to elucidate the exact causes of this effect may allow modeling of the change *in vitro*.

To the Breakout Group's knowledge, none of the cardiovascular toxicity models have been validated. After reviewing the literature, the likely candidate *in vitro* systems for an acute cardiotoxicity-testing scheme after chemical exposure could include the following:

- Short-term single-cell suspensions of adult rat myocytes to measure products of oxidation;
- Primary cultures of neonatal myocytes to measure changes in beating rates and plasma membrane potentials;
- Co-culture of smooth muscle cells or endothelial cells with macrophages, for example, to examine rate of wound healing (DNA synthesis);
- An immortalized cell line (e.g., the human fetal cardiac myocyte line) to measure classical cytotoxic endpoints.

It also may be important to include the perfused heart preparation, in spite of its limitations, for a comparison with the other *in vitro* models, because this system is the most representative of the *in vivo* situation.

4.8 *In Vitro* Methods to Study Hematopoietic Toxicity

Hematopoietic toxicity issues were recently reviewed by Gribaldo. [Progress in the Reduction, Refinement and Replacement of Animal Experiments, ed. M. Balls, A-M. van Zeller & M.E. Halder, pp. 671-677. Elsevier, Amsterdam, The Netherlands, 2000.] Xenobiotics can affect both the production and function of the various circulating cell populations, as well as the circulatory system that supports and helps maintain these cells. Acute effects on blood itself can also include the binding of materials to hemoglobin resulting in a loss of oxygen carrying capacity and cell lysis. Both of these latter endpoints should be easily modeled by *in vitro* systems if exposure conditions can be modeled.

During preclinical drug development it is often important to determine the following:

- Whether a new agent will be clinically toxic to the bone marrow cells;
- Whether the toxicity will be specific to one cell lineage (lymphocytes, neutrophils, megakaryocytes or erythrocytes);
- At what dose or plasma level the drug will be toxic;
- Which model best predicts the clinical situation, and
- When the onset and nadir of cytopenia and recovery will be likely to occur.

Validated *in vitro* tests using human cell systems are particularly important in this area as the prediction of human effects from animal systems are unreliable and necessitate the use of larger safety factors in human studies. *In vitro* colony-forming assays to study the growth and differentiation of various hematopoietic cell populations have been developed and perfected over the last twenty years, but none have yet been

validated for use in regulatory toxicology testing. A validation study of the use of colony-forming assays to test for the possible development of neutropenia is being supported by ECVAM. Methods to assess effects on thrombocytopoiesis and erythropoiesis are also available and can be considered for validation.

Associated projects have been also been carried out, such as the optimization of a protocol for detecting apoptosis using FACS analysis with fluorescent antibodies against Annexin V (Vermes et al., 1995). Using this assay, the induction of apoptosis in established stromal cells (SR-4897) (Pessina et al., 1997) and in murine and human leukemia cells (WEHI-3B; HL-60), following exposure to anti-neoplastic agents, has been investigated in relation to the cell cycle. The relationship between these observations and chromosome damage during mitosis is under evaluation. The drug sensitivities of myeloid progenitors from fresh murine bone marrow and from long-term cultures have been investigated by many authors including (Gribaldo et al., 1998a) as well as the role of the microenvironment in the modulation of anti-cancer drug activity (Pessina et al., 1999; Gribaldo et al., 1999).

In the session on hematotoxicity at the *3rd World Congress on Alternatives and Animal Use in the Life Sciences*, results were described for possible new endpoints (Balls et al., 2000). For example, the toxic effects of drugs on the proliferation of erythroblastic progenitors were evaluated using human and murine progenitors from long-term bone marrow cultures. Two kinds of tests were employed: (a) continuous exposure of human cord blood cells (CBC) and murine bone marrow cells (BMC) during the assay, and (b) pretreatment of long-term murine bone marrow cultures (for 24 hours and 96 hours), with subsequent testing of the clonogenic capacity of progenitor cells collected in the absence of the drug. The classes of drugs of interest in the study were: antivirals (3'-azido-3'-deoxythymidine), antidiabetics (chlorpropamide), and heme-analogous compounds (protoporphyrin IX/zinc [II]). The results indicate that all these drugs interfere with the normal hematopoietic process, causing a selective toxicity to the erythroid progenitors via

different mechanisms, and that human and murine progenitors have similar drug sensitivities. Moreover, the drugs exerted different toxicities based on the time of exposure.

Another aspect of hematotoxicology is in relation to the use of *in vitro* colony assays to support the risk assessment of industrial and food chemicals and pesticides. Some of these chemicals and formulations may interfere with the proliferative activity of the hematopoietic tissue and cause myelosuppression (Gribaldo et al., 1998b). One of the major difficulties in food toxicology is to establish the relationship between the consumption of a food contaminated by a toxin and the occurrence of a particular pathology. Clonogenic assays are a useful tool for establishing this relationship and for elucidating the mechanisms involved.

Three different clonogenic assays, with BFU-E (Burst-forming unit – erythrocytes), CFU-GM (Colony-forming unit granulocyte/macrophage), and CFU-MK (Colony-forming unit – megakaryocytes) cultures, have been used in toxicological investigations to detect or to confirm food-related hematotoxicity (Parent-Massin, 2000). By using these clonogenic assays, it has been possible to determine:

- The origin of neutropenia and hemorrhage induced by the consumption of trichothecene mycotoxin;
- The safety of a new process for manufacturing food additives;
- The mechanism of lead-induced hematotoxicity;
- The myelotoxicity of phycotoxins present in shellfish; and
- The risk to consumers and agricultural workers of hematological problems caused by pesticides (Parent-Massin and Thouvenot, 1995, 1993).

ECVAM is providing financial and organizational support to a new project on the development and prevalidation of *in vitro* assays for the prediction of thrombocytopenia. The continuous maintenance of an adequate supply of circulating

platelets is essential for sustaining life. Since neither platelets nor megakaryocytes are capable of regeneration, their production is dependent on a continuous generative process from self-replicating precursors. The CFU-MK is the progenitor cell thought to be immediately responsible for the production of megakaryocytes and is therefore being evaluated for its ability to predict thrombocytopenia.

Drug effects are by far the most common cause of platelet suppression in the bone marrow (Miescher, 1980). In many instances, thrombocytopenia is the first evidence of drug-induced toxicity, and continued administration of the drug produces total aplasia. Cytotoxic agents, such as 5-fluorouracil, vincristine, and cytosine arabinoside, cause perturbation of the bone marrow, with changes within the proliferating compartments, as well as effects on the maturing cell pool. In contrast, the thiazide diuretics, estrogens, and alcohol appear to have specific effects on platelet production. In addition, solvents, including benzene, insecticides (DDT, chlordane, lindane), spot removers, and model airplane glue, have all been associated with marrow-related thrombocytopenia (Amess, 1993).

Following bone marrow transplantation, the restoration of a normal platelet count occurs as a result of a compensatory adjustment in megakaryocytopoiesis (Vainchenker, 1995). For these reasons, appropriate *in vitro* endpoints for megakaryocytopoiesis that correlate well with platelet levels *in vivo* should be identified. A preliminary study carried out in ECVAM's laboratories to optimize an *in vitro* CFU-MK permitted a comparison of the suitability and drug-sensitivities of human BMC and CBC. The percentage of enrichment in CD34⁺/CD38⁻ cells from both populations was measured by using a negative selection system, and their clonogenicity was evaluated. Furthermore, the effects on megakaryocyte colony formation of busulphan, a cytotoxic drug, and the non-cytotoxic drugs, quinidine-sulphate, D-penicillamine, sodium valproate, and indomethacin were investigated by using both the whole cell populations and selected cells from the two sources. The data analyses confirmed the usefulness of the *in vitro* test as a

potential tool for screening drug toxicity to megakaryocyte progenitors. The *in vitro* test showed that human CBC can be used as a human target source, was more suitable for this purpose, and provided a means of avoiding ethical problems that exist in some countries connected with the collection of human BMC.

Up until now, primary cells have been more-reliable and more-relevant targets for clonogenic assays than the immortalized cell lines, but in the future, attempts should be made to establish standardized cell populations for *in vitro* tests, and in particular, for screening purposes. This may help to avoid the technical problems related to the absence of primary cell repositories, and to avoid the problem of inter-individual variability of the donors, in terms of drug sensitivity. A future topic will be the automated scoring of colonies in the clonogenic assays, which will provide the opportunity to refine the performance of the assays in terms of accuracy and repeatability, and to reduce personnel costs.

4.9 *In Vitro* Methods to Study Respiratory System Toxicity

The lungs fulfill the vital function of exchanging oxygen and carbon dioxide and a secondary function of protecting the organism from noxious or irritating inhaled stimuli. As such, the nasal and pulmonary airways represent a crucial organ-system that is likely to debilitate the organism if injured or irritated. The airways are particularly difficult to evaluate in *in vitro* because of their complexity. The following is a discussion of relevant airway cells and target-specific endpoints that should be considered in an *in vitro* battery for target-specific acute toxicity.

4.9.1 Cell Types

The tracheal-bronchial epithelial lining consists of stratified epithelium and diverse populations of other cell types including ciliated, secretory (mucous, Clara, serous), and non-secretory cells. The cells lining the airways may be represented by various human cell lines such as CCL-30 (nasal septum) (Poliquin et al., 1985) and BEAS-2B (bronchial-tracheal epithelia/transformed) (Noah et al., 1991; Reddel et al., 1988). More distally, alveolar Type II epithelia (A549)

function in conjunction with capillary endothelial cells for O₂:CO₂ exchange in the lower alveolar regions. This cell line can be used to show induction of P450 enzymes such as 1A1, 1B1, and 3A5 (Hukkanen et al., 2000), and to assess mucin production (Rose et al., 2000). The H441 cell line has been used in studies to evaluate toxicant effects on surfactant production *in vitro*. Various scavenger cells (alveolar macrophages) are present to engulf microbiological or foreign debris and destroy it. Several human alveolar macrophage cell lines exist which display the oxidative burst in response to irritants and biological debris (Marom et al., 1984). Neutrophils and eosinophils function as cellular sentinels of inflammation.

4.9.2 Endpoint Markers

A variety of endpoint markers valid for pulmonary cytotoxicity and irritation are available. ELISA-based assays can be used to quantitate many of these markers (e.g., cytokine, LDH), thus reducing the technical investment. The most useful markers will relate to the basic mechanisms by which airway epithelia respond to toxic exposure. LDH, a cytoplasmic enzyme released from damaged or lysed cells, is useful as a general marker of cytotoxicity. Mucus glycoprotein stain is a marker for alteration of mucous cells. Other possible endpoints include:

- Ciliary beat frequency (epithelial viability and function);
- Attachment (viability);
- Electrical resistance (to measure the integrity of the epithelial layer);
- Evans blue (to measure endothelial leakage);
- IL-8, IL-6, and TNFa (cytokine endpoints of inflammation).

As *in vitro* systems are developed and evaluated, biochemical markers of damage can be assayed in the lavaged fluid and directly compared to changes in similar markers in *in vitro* systems. Like the kidney, utilization of these comparisons will facilitate the development of predictive *in vitro* systems.

In vitro systems are available that can be used to indicate chemical-induced cell damage/death. The cells of the airways from animals or humans are relatively accessible to brushing, biopsy, and lavage, and therefore lend themselves for harvesting and use as primary cells (Larivee et al., 1990; Werle et al., 1994). Lung slices have been investigated for use in toxicology (Parrish, et al., 1995). The most useful markers are those that relate to the basic mechanisms by which airway epithelia respond to toxic exposure. However, most assays and cell models determining effects on special functions still need significant basic research before they will be useful as screening systems. The use of *in vitro* systems in respiratory toxicology was a subject of an ECVAM Workshop 18 (Lambre, et al. 1996).

4.10 Conclusions on the Use of *In Vitro* Systems for Assessing Organ-Specific Effects of Acute Exposure

There are significant ongoing advances in both technology and our understanding of biology that will have major effects on our ability to predict whole-animal (or human) toxic effects from non-whole animal model systems. For instance, toxicogenomics and proteomics provide rapid identification of early changes in cells *in vitro* or from individual animals and humans. However, these systems are very early in development and significant work will be needed to understand how the changes seen relate to whole animal toxicity, and particularly which changes are the direct result of exposure and which are due to secondary effects as the cells and tissues react to the primary injury. Because these systems appear to be very sensitive, it will also be important to determine how the assays can be used in the prediction of dose-response information for toxicology.

In recognition of the possible importance of advances in toxicogenomics to toxicology, the Breakout Group recommends that some effort be put toward preserving samples from animal studies for future evaluation so as to avoid having to repeat these studies at a future time.

It is very important that the proper quality control procedures be built into any *in vitro* test system developed for use in screening such as:

- Stability of the test material;
- Reactions of the test material with plastic in culture dishes and laboratory ware;
- Measurement(s) of test material concentration in the test vehicle;
- Non-specific binding to proteins in the culture medium;
- Reactive compounds;
- Ensuring that the cells reliably express the necessary metabolic systems.

Each individual test system will need to have a complete, standardized protocol developed, evaluated, and validated. All test schemes that are developed will then build on these validated tests. The prediction model for the entire scheme may also need to be evaluated and validated.

4.10.1 Proposed Scheme for Assessing Acute Toxicity Using Non-Whole Animal Methods

For the assessment of acute systemic toxicity for the purposes of setting hazard and risk levels for chemicals and products, data on specific organ toxicity are usually not needed. The need is for a system to appropriately classify the hazard of materials that may cause death after acute exposure irrespective of the specific organ damage. For such a system, the routine use of *in vitro* models to evaluate all possible organ effects would be impractical from both a time and money standpoint and evaluation of the effects of xenobiotics on specific organ function is not included in the current assays for acute toxicity. Current acute toxicity assay systems utilize young adult animals, often of only one sex, and only recognize observable effects within 14 days. Currently standard assays do not evaluate effects in different sub-populations or the long-term effects of single acute exposures.

Acute toxicity assays are primarily used to predict the toxicity of materials to humans. For this reason, where species differences are known, the Breakout Group recommends that screening

systems be developed that will predict effects in humans.

Breakout Group 3 discussed what additional assay systems would be required, in addition to the basic cytotoxicity assay discussed by Breakout Group 1, in order to replace the current acute oral toxicity assays for regulatory purposes. Breakout Group 3 developed a stepwise approach to address those effects identified in the discussions

of the specific organ systems that were highly relevant to the prediction of acute toxicity and would not be elucidated by a simple basal cytotoxicity test. This scheme is shown in Figure 4.1. The scheme includes a process for determining when additional specific effects need be evaluated, and gives some guidance on how to do so. The scheme includes steps proposed earlier by a expert workshop hosted by ECVAM and by Bjorn Ekwall in his series of papers.

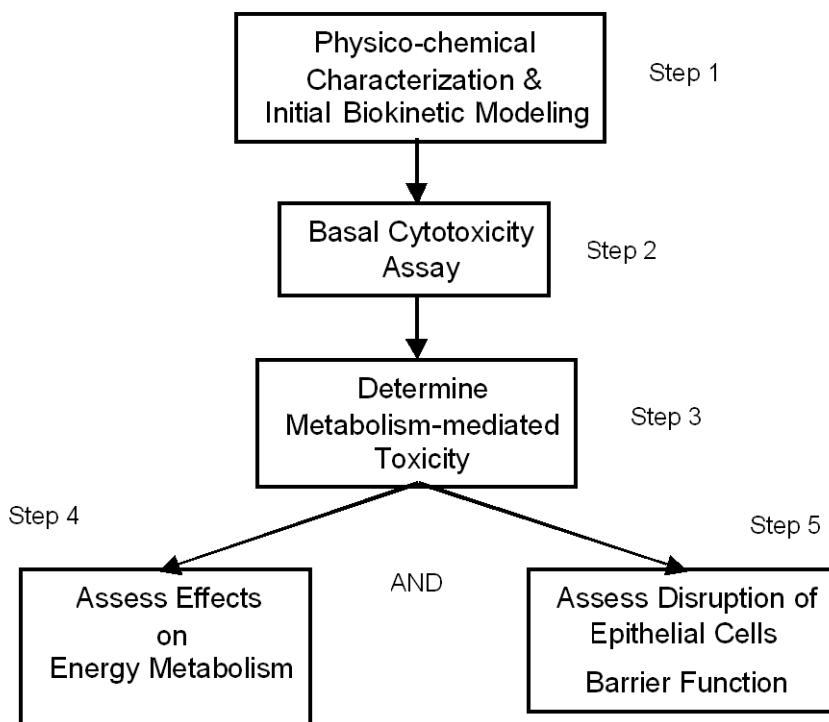


Figure 4.1 Proposed scheme for assessing acute toxicity using non-animal methods

(1) Step 1

- Perform physico-chemical characterization and initial biokinetic modeling (BG2 output). This information will be used for comparison with chemicals with similar structures or properties that have existing toxicity data. The information may also be useful in predicting organ distribution. It may

be possible to accurately predict the toxicity effects of some chemicals from this step alone.

(2) Step 2

- Conduct a basal cytotoxicity assay (BG1 output).

(3) Step 3

Determine the potential that metabolism will mediate the effect seen in Step 2:

- Use HEPG2 cells transfected with major metabolizing enzymes – at this time at least four different cell lines, each containing one of the four major metabolic enzymes will be needed. A secondary, and perhaps more relevant, possibility would be to use metabolically competent, primary human hepatocytes, but cell lines would allow a more standardized approach for regulatory purposes.
- Both cytotoxicity and, ideally, some measure of metabolism of the test substance, must be determined, either by detecting a decrease in the parent compound or by some method that directly detects metabolites.

A. If the material is more cytotoxic in the hepatocyte test system compared to that measured in Step 2, then assume the compound is metabolized to a toxic substance. In this case, the measure of cytotoxicity would use the value obtained from the metabolically active system instead of the value obtained in Step 2.

B. If the material is less cytotoxic than seen in Step 2, then it is assumed there is detoxification, and in those exposure scenarios where it can be shown the materials will pass through the liver before the rest of the body is exposed (first pass effect) it may be possible to reduce the prediction of toxicity accordingly.

C. If the cytotoxicity is similar to the basic cytotoxicity measured in Step 2, then the possibility of metabolite formation still must be

assessed to assure the metabolite will not have an effect on some other cells that do not have the metabolic capabilities of hepatocytes.

1. If there is no evidence of metabolism then the value used in Step 2 can be used.
2. If there is evidence of metabolism, Step 2 must be repeated after exposure to the metabolite(s) either by directly identifying the metabolites and using them in the system, or by some other undetermined systems such as co-cultures or conditioned media; exact protocols will need to be determined. The system that is developed must be able quantitatively assess the effects of the initial toxicant. For instance, according to Breakout Group 2, co-cultures will not enable the biokinetic modelers to predict systemic toxicity in a quantitative manner.

(4)

Step 4 (note: Steps 4 and 5 can be done in either order)

- Assess the test substance effect on energy metabolism by using a neuronal cell line that expresses good aerobic energy metabolism function. This system will help determine if the nervous or cardiovascular systems, both of which require high-energy metabolism, are likely target organs.
- The endpoints would be measurement of energy metabolism using a variety of specific probes of energy change, or oxygen consumption, or possibly mitochondrial function. The exact endpoint needs to be determined.

- If there is evidence of metabolism in Step 3, these tests must be done with both the parent compound and the metabolite(s).

(5) Step 5 (note: Steps 4 and 5 can be done in either order)

- Assess the ability of the compound to disrupt epithelial cell barrier function using a transepithelial resistance assay across a membrane, such as MDCK cells. The endpoint used could be dye leakage. This system will help in determining if organs dependent on epithelial barrier function for defense against toxic insult (e.g., brain, kidney) are likely target organs.
- If the compound causes disruption of barrier function at a value lower than the basal cytotoxicity, the endpoint used in determining the effect on the organism might need to be lowered to take this into consideration. [Note: Barrier disruption values will likely be lower than those that cause basal cytotoxicity.]
- If there is evidence of metabolism in Step 3, this test must be done with both the parent compound and the metabolite(s).

Next Steps

Before this system can be evaluated for implementation there is a need to:

- Identify the best cell culture systems to use based on accuracy, reproducibility, cost, and availability;
- Develop complete protocols for all the five steps and validate each assay;
- Develop prediction models for the prediction of relevant human toxic levels as required by regulatory agencies. Prediction of No Observed Adverse Effect Levels (NOAELs) would be addressed at this step;
- Evaluate the scheme with a number of test compounds covering all endpoints and then with enough compounds to develop a prediction model;
- Validate the entire scheme and prediction model.

The Breakout Group recommends that this work be done with the input and cooperation of the regulatory agencies and industries who have a need to use acute toxicity data in order to ensure the final result will meet everyone's needs.

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