2.0 ELEMENTS OF A STANDARD TEST FOR BASAL CYTOTOXICITY

It is likely that many different *in vitro* cytotoxicity methods could be used to help select the *in vivo* starting dose for an acute lethality assay. Two decades of experience indicate that *in vitro* basal cytotoxicity data determined in various primary cells, as well as in various permanent non-differentiated finite or transformed cell lines, generally show comparable cytotoxic concentrations of the same xenobiotic, regardless of the type of toxic endpoints investigated. The RC data, which consist of information from many different *in vitro* methods that vary in both cell type and cytotoxicity endpoint (i.e., specific protocol), indicated that exceptions to this "rule" were observed only for those chemicals (some insecticides, neurotropic chemicals, and chemicals requiring metabolic activation) that require specific cell types to express their toxicity (Halle, 1998). Thus, a recommendation cannot be made for the "most relevant" or "most typical" *in vitro* test for basal cytotoxicity.

Currently the ECVAM SIS lists 20 different test protocols for basal cytotoxicity. (Appendix B and http://www.ivtip.org/protocols.html#basalcyto.) Several *in vitro* tests listed in the SIS as "specific" for a certain purpose, such as prediction of eye and skin irritancy, in fact provide only basal cytotoxicity information.

Nonetheless, since the responsiveness of all cell culture test systems to xenobiotics can be influenced significantly by test design and culture conditions, there is a consensus among *in vitro* toxicologists to give preference to protocols that are highly responsive. For example, while increasing exposure times (e.g., from 1 hour [h] up to 48 h) will usually increase the responsiveness of the test, an increase in serum in the culture medium (e.g., from 5% up to 20%) will generally decrease the responsiveness of a cytotoxicity test.

2.1 Selection of Cell Lines / Cells

Analyses performed before or during the workshop (NIEHS, 2001) did not reveal significant differences between the basal cytotoxicity results obtained using permanent mammalian cell lines, primary human cells, or using the IC_{50X} approach of Halle and Spielmann (Halle, 1998; Spielmann et al., 1999; Halle and Spielmann, 1992). Thus, primary cells, as well as many currently available mammalian cell lines could be used, provided they are of sufficient quality to assure reproducibility over time. However, rodent (i.e., rat or mouse) or human cells are expected to be most useful for this approach. Established rodent cell lines are recommended because: 1) it is assumed that rodent cells would give the best prediction of rodent acute lethality, and 2) the use of a standard cell type for this *in vitro* cytotoxicity technique will hasten the generation of a database that can be used to analyze the usefulness of this approach. There are also arguments for utilizing human cell lines to assess basal cytotoxicity. For example, an analysis of the RC rodent acute lethality data relative to cytotoxicity data generated using human cell lines in the MEIC program showed that both were highly correlative (R^2=0.90) (NIEHS, 2001). A long-term advantage of using human cells is that the human cell cytotoxicity data derived from this approach can be added to human toxicity databases to facilitate the development of methods that may later predict acute human lethality.

Of the rodent cell lines used for basal cytotoxicity, the mouse fibroblast cell line BALB/c 3T3 A31 is probably the most frequently used. Thus, a stable background of historical data exists, including data from controlled and blinded validation studies (Gettings et al., 1991, 1992, 1994a, 1994b; Spielmann et al., 1991, 1993, 1996; Balls et al., 1995; Brantom et al., 1997). Other rodent cell lines that have been used in basal cytotoxicity assays are described by Clemedson et al. (1996).

Of the human cells used for basal cytotoxicity, NHK or fibroblasts are probably the cells most frequently used with good results in validation studies (Willshaw et al., 1994; Sina et al., 1995; Gettings et al., 1996; Harbell et al., 1997).

Fish cell lines or invertebrate cell lines are not recommended for determining basal cytotoxicity

(Ekwall et al., 1998). Although, according to the concept of basal cytotoxicity, they are expected to show failure of the same basic cell functions as mammalian cells would show at comparable chemical concentrations, it is not easy to create test designs that are highly responsive to xenobiotics. For example, due to doubling times of up to several days, the responsive growth inhibition protocol cannot be used easily.

Highly differentiated cells may not give the best prediction of acute lethality for the large variety of chemicals likely to be tested for acute toxicity (Ekwall et al., 1998). For example, to eliminate the possibility of metabolic activation or inactivation of chemicals, neither hepatocyte nor hepatoma cytotoxicity data were included in the RC. This does not preclude the use of hepatocytes in future studies, however, either to estimate cytotoxicity or to investigate the effect of metabolism or cell-specific toxicity (Seibert et al., 1996). Hepatocytes are essential to investigations of metabolism-mediated toxicity that will be required to meet the longer-term goal of replacing *in vivo* acute lethality testing with *in vitro* methods (Seibert et al., 1996).

Whether rodent or human cells are used, they should be capable of active division (population doubling time of approximately 30 h or less) so that chemicals that exert their toxicity primarily during cell division will be adequately detected in these relatively short-term assays. As described in Section 2.3, chemical exposure should last at least one full cell cycle.

Finally, selection of a cell line always should be made in the context of the intended cytotoxicity endpoint to be measured. For example, if NRU is the intended measurement endpoint, the cells used must possess a significant amount of lysosomes to incorporate neutral red dye. Embryonic stem cells, for example, do not contain the requisite organelles, and NRU cannot be used to determine cytotoxicity in these cells.

Both the mouse and human cells mentioned above are easily obtainable from commercial sources. Cytotoxicity data from both the BALB/3T3 A31 cell line and NHK cells are presented in Section 3.3 of this document as examples of how to qualify new cytotoxicity protocols for use with the RC method for predicting starting doses for acute lethality assays *in vivo*.

2.2 Recommended Measurement Endpoints for Basal Cytotoxicity

Many measurement endpoints for cytotoxicity are well established and have been used to assess basal cytotoxicity. For inclusion of IC_{50} values in the RC, the following endpoints were accepted as sufficiently characteristic of basal cytotoxicity (Spielmann et al., 1999; Halle, 1998):

1) Inhibition of cell proliferation:
- Cell number
- Cell protein
- DNA content, DNA synthesis
- Colony formation

2) Cell viability - metabolic markers:
- Metabolic inhibition test (MIT-24)
- Mitochondrial reduction of tetrazolium salts into insoluble dye (MTT test), or, more recently, into soluble dye (MTS test or XTT test [e.g., "EZ4U"]).

3) Decreased cell viability - membrane markers:
- NRU into cell lysosomes
- Trypan Blue exclusion
- Cell attachment, cell detachment

4) Differentiation markers
- Functional differentiation within cell islets
- Morphological differentiation within cell islets
- Intracellular morphology

Markers of the release of intracellular components, such as the enzyme lactate dehydrogenase (i.e., LDH release test), or of dye introduced into the cells previous to chemical exposure (e.g., fluorescein leakage [FL] test or Neutral Red Release [NRR] test) were not considered to be characteristic for basal cytotoxicity because they specifically detect damage of the outer cell membrane and generally are associated with short-term chemical exposure. A chemical that specifically damages only cell membranes, however, will be detected correctly in one of the tests for basal cytotoxicity listed above.

2.3 Recommendations for Cytotoxicity Test Protocols

Since the RC was constructed with data from many different *in vitro* protocols, a number of different *in vitro* cytotoxicity protocols might produce correlations with *in vivo* acute lethality similar to the correlation produced by the RC. It is strongly suggested, however, that any proposed *in vitro* protocol incorporate the following conditions:

(a) Use a cell line (or primary cells) that divides rapidly with doubling times of less than 30 h under standard culture conditions, preferably with normal serum types, e.g., calf serum (CS), newborn calf serum (NBCS), or serum-free medium.

(b) Use only cells in the exponential phase of growth. Never use cells immediately after thawing them from frozen stock. Allow cells to grow 1-2 passages before they are used in the cytotoxicity test.

(c) The chemical exposure period should be at least the duration of one cell cycle, i.e., 24 – 72 h (Riddell et al., 1986).

(d) Initial seeding should be done at a density that allows rapid growth throughout the exposure period.

(e) Use appropriate positive and vehicle control materials for which cytotoxicity, or lack of cytotoxicity, has been well characterized by the performing laboratory.

(f) Use solvents only at levels previously shown not to cause cytotoxicity to the cell system over the entire period of the assay.

(g) Use a measurement endpoint that is well established and that has good interlaboratory reproducibility. Give preference to endpoints that determine either cell proliferation or cell viability (e.g., NRU, MTT, XTT). Simple endpoints such as total protein content are not recommended, as they may under-predict the toxicity of certain test chemicals by staining dead cells.

(h) The protocol should be compatible with 96-well plates and apparatus such as spectrophotometers that allow a quick and precise measurement of the endpoint.

(i) Complete a detailed concentration-response experiment using a progression factor that yields graded effects between no effect and total cytotoxicity. Any desired toxicity measure can be derived from a well-designed concentration-response experiment. Experiments that seek to detect only a marker concentration, such as the highest tolerated dose or the lowest cytotoxic dose, are characterized by a lack of information and a low level of accuracy.