Short-term Toxicity Tests for Non-genotoxic Effects
Edited by P. Bourdeau *et al.*

CHAPTER 7

Toxicity Tests with Mammalian Cell Cultures

B. EKWALL, V. SILANO, A. PAGANUZZI-STAMMATI AND F. ZUCCO

7.1 INTRODUCTION

Cell culture can be used to screen for toxicity both by estimation of the basal functions of the cell (i.e. those processes common to all types of cells) or by tests on specialized cell functions (Ekwall, 1983b). General toxicity tests, aimed mainly at detection of the biological activity of test substances, can be carried out on many cell types (e.g. fibroblasts, HeLa and hepatoma cells). A number of parameters including vital staining, cytosolic enzyme release, cell growth and cloning efficiency are used as end-points to measure toxicity. Organ-specific toxic effects are tested using specialized cells by measuring alterations in membrane and metabolism integrity and/or in specific cell functions (e.g. glycogen metabolism in primary hepatocyte cultures, beating rate in mixed myocardial cells or myocytes, and phagocytosis in macrophages).

Major problems in the interpretation of results obtained *in vitro* to identify cell-specific effects are as follows:

(1) Since basal cell functions always support specific cell functions, chemicals that are capable of affecting basal cell functions are also likely to affect the specialized ones;
(2) The effects of a test substance on a cell system may be different depending on the conditions of incubation (e.g. incubation time and concentration of toxicant). Therefore, unless a set of favourable circumstances occurs and a well-planned experimental design is adhered to, it may prove difficult to distinguish between basal and organ-specific effects.

Cytotoxicity tests using specialized cells have proved most useful when the *in vivo* toxicity of a chemical is already well established and where *in vitro* investigations using specialized cell cultures have been used to clarify the mechanisms of toxic action on the target tissue. These tests have also provided useful insight into the pathogeneses of some human diseases (e.g. for liver diseases, see Klaassen and Stacey, 1982, and for coeliac disease, see Auricchio *et al.*, 1985).

The assessment of the significance of the results of *in vitro* tests in relation to the *in vivo* situation presents another major problem. Much can be learned from past experience with drugs which indicates that intrinsic cell sensitivity is only one factor, and not necessarily the most important, in determining specificity of toxic action of chemicals. Other factors, more directly related to chemical kinetics such as rates of absorption, biotransformation, distribution and excretion, which influence the exposure at the level of target cells *in vivo* cannot, at present, be adequately simulated *in vitro*. Furthermore, even when the appropriate cell type is used, intrinsic cell sensitivity depends on a number of cell characteristics which are likely to be preserved only in part *in vitro*; these include chemical biotransformation and binding, membrane permeability characteristics and surface determinants, intracellular synthetic pathways and adaptive and recovery mechanisms. For some toxic chemicals, it is the functional status of the cell rather than the cell type that determines the extent to which the inhibition of a given biochemical mechanism is critical to the function and survival of the cell.

For a more detailed discussion of cytological and biochemical differences responsible for selective toxicity of chemicals, the textbooks by Albert (1979) and Schwartz and Mihic (1973) can be consulted. Some anti-cancer drugs will be discussed here to illustrate the biological base for selective toxicity and some uses and limitations of *in vitro* cytotoxicity testing. Cancer cells are normally highly-specialized cells which have regressed to a much simpler, more primitive stage and which, unlike the normal parent, divide continuously, although inefficiently. Because a much higher proportion of cancer cells are undergoing active division, they are more vulnerable than most normal cells to anti-cancer drugs. However, normal tissues with high mitotic indices (e.g. bone marrow, spleen, thymus and intestinal epithelium) are also more susceptible to anti-cancer drugs. Both in normal and in neoplastic proliferating tissues, the toxicity of many of these drugs appears to be related to effects on the mitotic spindle and replicating DNA. Chemicals capable of a direct attack on the microtubules or the mitotic spindle (e.g. colchicine or the vinca alkaloids) are selective for proliferating cells, but only rarely are they more selective for tumour tissues. The selective toxicity on tumours observed for some of the chemotherapeutic agents, depends more on pharmacokinetic and metabolic factors in target cells than on the direct proximal action of the agent. The alkylating and intercalating drugs, which act directly at the level of DNA, and the mitotic poisons have been shown to produce a fairly uniform response in mammalian replicating cells, i.e. clumping and fragmentation of the chromatin (Schwartz and Mihic, 1973). Differential cell lethality in the presence of alkylating agents is probably due to differences in repair mechanisms as related to the cell's demand for functional DNA (Alexander, 1969). Thus, a slow rate of repair in rapidly dividing cells will be more critical than the same repair rate in slowly dividing or non-proliferating cells. A number of DNA antimetabolites, co-factor analogs and enzyme inhibitors, which inhibit enzymes involved in the synthesis of DNA or its precursors, cause characteristic lesions in proliferating cells with a scatter of toxicity in non-proliferating

ones. In many cases, the selectivity of action of these agents depends mainly on the fact that normal proliferating tissues have distinctive physiological or biochemical characteristics that affect drug actions (Schwartz and Mihic, 1973).

For the sake of completeness, it should be mentioned that, in addition to cytotoxicity testing, cell culture systems are also useful to carry out metabolism studies including biotransformation, interaction with endogenous metabolites, binding to cells, and induction of metabolism.

This chapter is an overview of the present state of the art of *in vitro* testing of cell toxicity of chemicals. Available cell culture systems and methodologies are discussed in the light of present experimental trends. Furthermore, the significance of cell systems as alternatives to whole animal systems for predicting toxic potential of chemicals with respect to selected end-points and for selecting priority among existing chemicals are examined together with possible future developments.

7.2 CELL CULTURE SYSTEMS AND METHODS

The growing use of *in vitro* systems in biomedical research has accentuated the need for standardization and clarification of the terms more frequently used by researchers working in this field. In 1964, a Terminology Committee of the Tissue Culture Association was set up in order to recommend a generally acceptable terminology. The final report of the Committee was accepted at the annual meeting of the Tissue Culture Association in 1966 (Fedoroff, 1966). This nomenclature was subsequently revised in 1984 (Schaeffer, 1984). Primary cell cultures, cell lines and cell strains have been defined as follows:

- A primary cell culture is 'a culture started from cells, tissues or organs taken directly from organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a cell line'.
- A cell line 'arises from a primary culture at the time of the first successful subculture. The term, cell line, implies that cultures from it consist of numerous lineages of cells originally present in the primary culture. The terms, finite, or continuous, are used as prefixes if the status of the culture is known. If not, the term line will suffice'.
- A cell strain 'derives either from a primary culture or a cell line by the selection or cloning of cells having specific properties or markers. The properties or markers must persist during subsequent cultivation'.

Cell lines may be finite or continuous. A finite cell line is generally diploid and, in this case, no less than 75 per cent of all the cells must be of the same standard karyotype as the parent species; its lifespan is approximately 40–50 divisions (Fedoroff, 1966; Hayflick and Moorhead, 1961). A continuous cell line derives from primary cultures or diploid cell lines by transformation processes which are either spontaneous, or induced by viruses, chemical or physical agents (Fedoroff, 1977).

When a cell line derives from a single cell, it is called a clonal cell line. Clonal cell lines can be obtained by several techniques, starting from primary cultures, diploid cell lines or established cell lines. They are not necessarily homogeneous populations and only frequent cloning can keep culture heterogeneity to a minimum.

Available cell lines are collected by the American Type Culture Collection which provides a catalogue listing of every cell type with its history and information concerning viability, growth medium, growth characteristics, plating efficiency, age of culture since origin, morphology, karyology, sterility tests and virus susceptibility.

Primary cell cultures have morphological and biochemical characteristics that are more similar to those of the original tissue; however, problems with obtaining reproducible results may negate these advantages. Nevertheless, primary cultures offer the only possibility for comparative studies of some specialized tissues taken from different animal species where cell lines and strains from the same tissues are not available. Primary cultures are generally more sensitive to the effects of toxic chemicals than are cell lines because, while exposed, they have also to adapt to culture conditions. The main limitations of primary cultures are low homogeneity and a tendency to rapid loss of specialization under culture conditions. Cell lines offer the advantage of being more homogeneous and standardized than primary cultures. They are well characterized, easy to cultivate and reproducible results are easier to obtain. On the other hand, they may be quite different from the original tissue due to the fact that established cell lines have undergone a number of transformations.

Cell strains have the advantage of being more homogeneous populations from the point of view of selected characteristics, but they present the same disadvantages as cell lines from which they derive.

Compared with cells from normal adult tissues, embryo and tumour cells are more easily cultured because they have a higher growth capability and adapt more readily to variations in external factors. The setting up of primary cell cultures and, to a larger extent, of continuous cell lines imply some loss of differentiation, but there are many cell types that display highly specialized biological activities *in vitro* that are characteristic of their original tissues or organs (Sato and Yasumura, 1966). Some examples were reported in the review of Paganuzzi-Stammati *et al.* (1981). A growing number of cell types have been shown more recently to retain some specialized functions in culture. Some examples include:

(1) Endothelial cells *in vitro* display several specialized functions including a non-thrombogenic surface to platelets, Factor VIII antigen, the surface angiotensin-converting enzyme, and synthesis of fibronectin and collagen (Striker *et al.*, 1980).
(2) Adult cardiac myocytes from different animal species retain several biochemical, morphological and physiological characteristics (Lieberman *et al.*, 1980).
(3) Human epidermal keratinocytes are capable of terminal differentiation in

culture; in particular, they produce keratin and the cells of the upper layer of the colonies lose their ability to divide and develop a cornified cell envelope.

(4) Mouse secondary cultures of Schwann cells are still able to synthesize enzymes typical of myelin-forming cells (White *et al.*, 1983).

Some tumour cell lines can, under some culture conditions, retain a degree of differentiation *in vitro*. Engvall *et al.* (1984) obtained sublines from mouse teratocarcinoma-derived endodermal cell line PF Mr-9, that possess a number of protein markers of the parent cells, for example, the two intermediate filament proteins Endo A and B. They also produce a large amount of laminin and a small amount of fibronectin as well as Type IV collagen and heparan-sulphate proteoglycans.

New and sophisticated *in vitro* techniques are very useful to maintain the specialized functions of cells. Among these is the use of serum-free media consisting of a nutrient basal medium supplemented with hormones and growth factors necessary to the various cell types.

These selective media facilitate adaptation of cells to the culture and allow a better standardization of experimental conditions. Serum is a very complex and poorly characterized mixture, the composition of which may vary according to the commercial batch; some components essential for cell growth may be absent. Serum may contain naturally-occurring substances (Barnes and Sato, 1980b) or microbiological contaminants (e.g. mycoplasma, viruses, endotoxins) that are toxic for certain types of cultures (Higuchi, 1976).

Serum-free media facilitates the isolation of the desired cell type and, in setting up primary cell cultures, they almost completely eliminate the overgrowth of fibroblasts that, usually, grow rapidly in serum-supplemented media. Moreover, these selective media are useful to study interactions of cells with hormones or drugs and to perform cell nutrition studies (Barnes and Sato, 1980a). Examples of the advantages of serum-free (or low serum) media include the establishment of differentiated rat thyroid cells in hormone-supplemented medium containing only very small amounts of serum (Ambesi-Impiombato *et al.*, 1980). Other cell types can also be grown more efficiently and in a more differentiated way in serum-free media. For example, the MC84 5 line forms villus-like secretory structures (Murakami and Masui, 1980), the HLE 222 human lung epidermoid carcinoma cells produce extensive keratinization (Barnes *et al.*, 1980), rat granulosa cells synthetize large amounts of progestins and oestrogens after stimulations by follicle-stimulating hormone (FSH) (Orly *et al.*, 1980); additional examples are reported by Barnes and Sato (1980a).

Much attention has been given to the identification of factors such as hormones, growth factors, binding proteins, attachment and spreading factors, which are essential for the replacement of the various functions carried out by serum. Some (e.g. insulin, transferrin) are common to most cell types whereas others are specific for particular cultures (Barnes and Sato, 1980b). Often the various factors effective for

a given cell line have also been shown to be useful for the primary culture from the same tissue (Barnes and Sato, 1980b). Many investigations have been devoted to the development of selective media for primary cultures (Sato *et al.*, 1982).

Cell lines are more widely used for general toxicity studies than primary cell cultures because they are well characterized and more easily cultured. The more commonly used cell lines include diploid human fibroblast lines (e.g. WI-38) and tumour cell lines (e.g. HeLa). When the mechanism of toxicity of a chemical is under investigation and it becomes necessary to take into account specific characteristics of specialized cell types, primary cell cultures of the target organ or tissue are often used in conjunction with cell lines from the same origin.

7.3 *IN VITRO* TESTING OF CELL TOXICITY OF CHEMICALS: METHODOLOGICAL ASPECTS

The first and most readily observed effect following exposure of cells to toxicants is morphological alteration in the cell layer and/or cell shape in monolayer culture. Therefore, it is not surprising that morphological alterations are used as an index of toxicity. A systematic appraisal of cell injury has been attempted to allow a greater standardization of the observations. A checklist suitable for computer-based programmes has been proposed (Walton and Buckley, 1975; Walton, 1975). Different types of toxic effects may require investigative tools of different levels of sensitivity. Gross modifications such as blebbing or vacuolization can be observed using light microscopy (Ekwall, 1983b) whereas fine ultrastructural modifications require analysis by transmission or scanning electron microscopy.

Another indicator of toxicity is altered cell growth. The effect of chemicals on the capability of cells to replicate is used as an index of toxicity; the concentration of the substances at which 50 per cent of the cells do not multiply is called the median inhibitory dose (ID_{50}). A more specific measure of replication is plating efficiency—the ability of cells (100–200 per dish, 60 mm diameter) to form colonies after 10–15 days of culture in the presence of a toxic agent gives more complete information, indicating both cell survival and ability to reproduce (Nardone, 1977). Cell reproduction can be measured by several parameters including cell count, DNA content, protein content, or enzyme activity (e.g. ornithine-decarboxylase; Costa, 1979). Each of these parameters can be measured by more or less sophisticated means. Examples are the assay of DNA content by biochemical methods and incorporation of radiolabelled precursors.

Another crude index of toxicity is cell viability measured by using vital dyes such as trypan blue which enters dead cells only or neutral red that is actively taken up by living cells; the latter is commonly used in biomaterial testing by the agar overlay method (Guess *et al.*, 1965). A count of dead and vital cells in comparison with the control provides an index of lethality of the test compound. The release of ^{51}Cr is another index of lethality measuring membrane functions (Holden *et al.*, 1973).

Other indices of toxicity to basal cell functions involve measurement of biochemi-

cal or metabolic cell alterations. The pathways of energy transmission and their alterations, O_2 consumption or ATP levels are usually measured by the Clark electrode (Harmon and Sanborn, 1982; Yoshida *et al.*, 1979) and by the luciferin–luciferase assay respectively (Waters *et al.*, 1975). High pressure liquid chromatography has been used to measure the pool of DNA and RNA precursors whose imbalance is considered an important toxicity indicator (Bianchi *et al.*, 1982; Bianchi, 1982). Acid phosphatase activity has been used as an index of cell damage (Bitensky, 1963). Lactate dehydrogenase activity in the culture medium, usually measured as the NADH–NAD conversion needed to convert pyruvate into lactate (Elferink, 1979; Acosta *et al.*, 1978), has been used as an index of membrane damage.

Cells derived from different organs or tissues, that retain some specialized functions *in vitro* or that maintain specialized structures, have also been widely used in toxicology. For these cells, effects on more specialized functions and/or structures have usually been taken into account in addition to effects on basic ones; specific end-products, metabolic pathways, membrane functions or structures have been tested. A tentative grouping of possible specific end-points used for some cell types is shown in Table 7.1.

Table 7.1 End-points more commonly used as markers of toxic effects in specialized cells*

Synthesis or release of specific molecules Collagen mat, heme, haemoglobin, albumin, urea, lipoprotein, α-aminolevulinic acid, bile salts, metallothionein, glycosaminoglycans, proline and hydroxyproline, energy-dependent choline accumulation, histamine release and c-AMP.
Synthesis, activity or release of specific enzymes β-glucuronidase, lactate-dehydrogenase, oubain-insensitive ATPase, G-6-P dehydrogenase, ASAL, glycogen phosphorylase, glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, acetylcholinesterase, and renin.
Interactions of compound with cells Phagocytosis, cytoplasmic inclusions, intracellular accumulation, siderosome formation, uptake and/or binding of compound to cytosol and lipoproteins, mitogenic response.
Alterations of metabolic pathways Methaemoglobin reduction, glucose-transport, 5-methyltetrahydropholate accumulation, hormone-stimulated gluconeogenesis, lipid peroxidation, fat accumulation and glucosamine and galactose incorporation.
Cell surface activities Adhesiveness, Con-A agglutination, antibody-mediated rosette formation, complement deposition on treated cell membrane, chemotactic migration, antagonism with Histidine-H1 receptor, GABA-mediated postsynaptic inhibition, spike frequency, membrane polarization, fibre retraction or outgrowth, and electrophysiological alteration.

* For references see Paganuzzi-Stammati *et al.* (1981).

Because some cell systems do not possess an efficient metabolism (see also Section 7.4), *in vitro* testing may require some form of metabolic activation. This is usually done in one of three ways:

(1) By the addition of S-9 fraction from rat liver; usually the mixed function oxidases are pre-induced by treating the animals with phenobarbital, β-naphthaflavone or Aroclor (Dolfini *et al.*, 1973);
(2) By pre-incubation of the test substance with a primary hepatocyte culture and addition of the pre-incubated medium to the test culture (Moldeus *et al.*, 1978).
(3) By co-culture of the target cell with hepatocytes in the presence of the test substance (Grisham, 1979).

The physicochemical properties of test compounds determine exposure conditions and the concentration of toxic agents in the culture medium. The medium is usually an aqueous saline solution with the addition of serum at concentrations ranging from 2 to 15 per cent. Only hydrophilic test compounds can be completely solubilized. For gaseous toxic agents, special incubation equipment is available to ensure a constant exposure with time provided the partition coefficients and solubilities are known. Similar problems apply to hydrophobic test compounds or mineral particulates. Lipophilic substances can be solubilized in ethanol, methanol or dimethylsulphoxide before addition to the medium; a control using the carrier solvent alone must be used.

In order to increase comparability of results and optimize testing procedures, standardization and harmonization of the experimental approaches and procedures is desirable. The use of well characterized cell lines, possibly of a human origin, is necessary and basic and specific toxicity end-points, as well as the most suitable assay methods, should be agreed upon. Cell cultures should be examined periodically for possible contamination with micro-organisms or for cross-contamination with other mammalian cell types. Periodic checks of the karotype would be appropriate. Reports should provide details of exposure conditions including information on the purity of the test compound. Moreover, the measurement of the concentration of the compound at the beginning and at the end of the experiment (and the reporting of concentrations as 'molarity', whenever possible), would simplify the comparison of results from one laboratory to another.

7.4 PRESENT TRENDS OF TOXICOLOGY INVESTIGATIONS WITH CELL CULTURE SYSTEMS

A literature survey on present trends in toxicology investigations using cell cultures has been published (Paganuzzi-Stammati *et al.*, 1981). These authors identified three major research areas where cell culture systems have proved to be extremely useful: (1) clarification of action mechanisms of toxic substances with specialized

cell systems; (2) clarification of the effects on basic cell functions mainly with fibroblasts and epithelioid cells; and (3) in metabolism investigations.

7.4.1 Investigations with specialized cell systems

Several specialized cell types have been used in toxicology investigations. Among the more widely used are cells derived from liver, lung, heart, muscle and nervous and reticuloendothelial systems (Table 7.2). Primary cultures or isolated cells are widely used because the cells retain their specialized functions better.

An examintion of publications since 1981 did not reveal major new trends although some interesting new approaches were noted.

Interaction of xenobiotics with nerve-growth-factor induced fibre outgrowth in nervous cells have been investigated (Nakada *et al.*, 1981). Some studies on the synthesis of prostaglandins (Burstein *et al.*, 1983) and alterations in oxidative response have been recently reported in macrophages (Castranova *et al.*, 1980; Hoidal *et al.*, 1981; Williams and Cole, 1981; Garrett *et al.*, 1981) and more attention has been recently paid to peroxidation and effects on phospholipase in hepatocytes (Stacey and Klaassen, 1981a, 1981b; Stacey *et al.*, 1982; Lamb and Schwartz, 1982). Substances that have been most frequently studied are generally already well known for their systemic effects on specific organs or tissues (e.g. pesticides on nervous systems and liver cells, dusts on macrophages, metals on liver and kidney cells).

7.4.2 Investigations with non-specialized cells

These investigations have been based on fibroblast, epithelioid and other cell lines.

Table 7.2 Specialized cells commonly used in toxicology

Organ of origin	Primary cultures or isolated cells	Cell lines
Nervous system	Chick embryo ganglia; chick embryo brain cells; mouse and rat cerebellum cells	C 1300 (mouse); C 6 (rat)
Lung	Human, rabbit and rat alveoloar macrophages	P 388D1 (mouse); A 549 (human)
Reticuloendothelial system	Human, mouse lymphocytes and erythrocytes; rat and mouse peritoneal macrophages	—
Liver	Rat and chick embryo hepatocytes	Chang (human); CC1144 (rat); ARL (rat); RLC-GA (rat)

The aim has been to study the effects of test substances on structures and functions common to most types of cells, i.e., basal cytotoxicity (Ekwall, 1983b). Substances under investigation mainly have been chemicals subject to registration procedures in many countries such as drugs and pesticides. Effects have been studied by monitoring rather simple parameters such as cell growth or viability on cell lines such as HeLa, CHO, 3T3, WI-38, human skin fibroblasts and BHK. Some of these investigations have been undertaken to validate the use of the cell systems as screening procedures. Such studies will be described in more detail in Section 7.5.

7.4.3 Metabolism investigations

Metabolism has been investigated *in vitro* with systems of different complexity by using, for instance, liver slices, hepatocytes or purified microsomes. Liver slices or hepatocytes have been extensively used in the investigation of metabolic pathways, and in the identification of intermediates or secondary products. A number of investigations have been devoted to the preparation of isolated hepatocytes preserving their metabolic activities. Recently, the very rapid decay of Phase I enzymes in culture systems has been prevented by using special media; monoxygenase levels were kept near to the values *in vivo* for quite a long time (Grisham, 1979). Microsome preparations, first introduced for mutagenicity assays, have also been used as activating systems in cytotoxicity investigations; however, they may themselves contribute some cytotoxic activity (Balls and Bridges, 1983). Although the importance of metabolism in liver cells is well known, cell types derived from other organs or tissues such as lung (Baird *et al.*, 1980; Tell and Douglas, 1980), aorta (Baird *et al.*, 1980) intestine (Schiller and Lucier, 1978), and ovary (Drake *et al.*, 1982) also display metabolic activities which may have a major significance in determining toxicity. Phase I and II metabolic reactions have been studied *in vitro* by cocultivating human adult hepatocytes and rat liver epithelial cells for several weeks (Begue *et al.*, 1983).

It is well known that metabolism may differ significantly among various animal species (Miller and Miller, 1971; Weisburger *et al.*, 1964; Quinn *et al.*, 1958; Hucker, 1970). For this reason, it is highly desirable to use human cells for metabolism investigations. However, because of difficulties associated with the availability of material and standardization of techniques, only a few papers have been published in this area (Guillouzo *et al.*, 1982; Guguen-Guillouzo *et al.*, 1988).

7.5 CELL SYSTEMS AS ALTERNATIVES TO WHOLE ANIMAL SYSTEMS FOR PREDICTING TOXIC POTENTIALS OF CHEMICALS WITH RESPECT TO SELECTED END-POINTS

The objectives of toxicity tests, whether conducted in the whole animal or in cell systems, must be to predict the adverse effects of the tested compound in human beings both in a qualitative and quantitative way. To this end, both animal and

cellular tests try to simulate aspects of the human body. The animal is a more complete model of the human body, which includes basic and specific cell functions as well as almost all human organizational functions (Ekwall, 1983b), including pharmacokinetic determinants. The cell culture is a model of a target tissue in the human body and mimics the response of human cells to exposure to chemicals. Provided that time and degree of exposure (dosage for animals and concentration/exposure time in cell tests) in the experiments correspond to human exposure, both models can potentially predict any type of chemical interference with corresponding aspects of the human body.

There are interesting differences between whole animal and cellular systems with respect to the different types of toxic action measured. In principle, the whole animal model measures the critical toxicity of a chemical, i.e. the one or two toxic effects that appear first when a dose to an animal is gradually increased. These critical effects often overshadow many other potential toxic effects, which are thus not recorded by the whole animal experiment. Relatively subtle species differences in receptor affinity or metabolic pathways may influence which of the many kinds of potential effects are observed so that whereas animal experiments have the advantage of predicting, in many cases, the critical effects in the human body, they could fail to do so because of species differences. Cell cultures, on the other hand, will only measure potential toxic effects. The critical toxicity for the human body must then be judged by a comparison of tissue culture results with actual human exposure to the chemicals. In the case of local toxicity, this may be relatively simple but in the case of systemic toxicity, concentrations of the toxicants in human tissues or the dose and the pharmacokinetics of the compounds must be known. However, for screening purposes, it will suffice to compare the cell toxicity with possible exposures to chemicals *in vivo*.

Tissue culture tests will not reveal toxicity due to disturbance of extracellular, organizational functions in the human body. Therefore, they must remain complementary to whole animal tests. The extent to which they can be relied upon as alternatives to animal screening tests depends on how frequently chemicals affect organizational aspects of the body. As with animal tests, most tissue culture tests of acute toxicity are optimal in the sense that they involve concentrations and exposure times directly transferable to the human condition in contrast to the case for the short-term mutagenicity and carcinogenicity screening tests (Bartsch and Tomatis, 1983).

There are two types of cellular models, i.e. undifferentiated and differentiated cells, used in acute toxicity testing (Ekwall, 1983b). Of these two types, the simple systems measuring basal cytotoxicity are probably the more useful in the sense that a central toxic effect is measured. This type of test is multi-purpose, and can be used to test both essential traits of local toxicity as well as various forms of systemic toxicity, including teratogenicity. The use of specialized cells for screening purposes will probably be determined by the cost and benefit of each model. Further research on differential cytotoxicity (comparison of results from parallel testing of substances in different cell systems, e.g., a cell line and hepatocytes as described by

Ekwall and Acosta, 1982) will show how often local irritancy to corneal, gingival or dermal cells actually is caused by selective cytotoxicity, not measured by basal cytotoxicity tests. Likewise, the frequency of organ-specific cytotoxicity to the liver, nervous system (Nardone, 1983), kidney, and so forth, will be determined. Until the frequency of selective cytotoxicity to different organs is fairly well known, the use of organ-specific tests for screening purposes is difficult to define (Ekwall and Ekwall, 1988).

The potential for cytotoxicity tests to supplement or provide an alternative to the use of laboratory animals in toxicity investigations has been discussed recently (Lindgren *et al.*, 1983; Williams *et al.*, 1983; Goldberg and Liebert, 1983; Balls *et al.*, 1983). Their use in range-finding and screening tests is tending to increase for scientific, ethic and economic reasons. Some current trends are described in the following paragraphs.

7.5.1 Implants

The most traditional use of tissue cultures in toxicology is the testing of implants, including dental materials for local irritancy (Ekwall, 1980; Browne and Tyas, 1979; Kasten *et al.*, 1982). Two studies (Hensten-Pettersen and Helgeland, 1977, 1981) compared different systems and cell types (including cell lines and secondary cultures of human gingival fibroblasts) in the prediction of local irritancy. Differences in methods (rather than in cell types used) caused differences in the results obtained; predictability was generally low; the reason may be the attempt to extrapolate from short-term incubation *in vitro* to long-term effects *in vivo* and the fact that true concentrations were not tested and that circulation may rapidly remove some substances from the local site *in vivo*. Because of the unreliability of various methods to produce comparable results, the US Commission on Dental Materials, Instruments and Therapeutics (COMIET) recommended the use of three standard tissue culture tests in 1978 (Stanford, 1980). These methods are a ^{51}Cr-release method (Spangberg, 1973), a millipore filter test (Wennberg *et al.*, 1979), and the widely-used agar-overlay test (Autian, 1977). These recommendations are now followed by all professional workers involved in testing dental materials. This must ultimately lead to long series of compounds tested by each method, a prerequisite for large-scale evaluation of results.

7.5.2 Eye and skin irritants

The Draize test to assess the local irritancy of chemicals in the rabbit eye has been criticized for cruelty and the subjectiveness of scoring, the latter probably contributing to the large inter-laboratory variability of results (Williams *et al.*, 1982; Wahlberg, 1983; Swanston, 1983). Alternative tests with enucleated rabbit eyes (Burton *et al.*, 1981; York *et al.*, 1982) or with rabbit ileum (Muir, 1983; Muir, 1984) have been proposed. Trials using culture tests for eye irritancy testing have

given equivocal (Krejci, 1976; Scaife, 1983) or clearly positive results (North-Root *et al.*, 1982; Kemp *et al.*, 1983a; Kemp *et al.*, 1983b; Shopsis and Sathe, 1984; Borenfreund and Borrero, 1984; Selling and Ekwall, 1985). Borenfreund tested 34 toxicants in an assay using a number of cell types, including epithelial rabbit corneal cells, and found the same degree of toxicity in all cell systems tested. It seems that permanent cell lines are as good indicators of eye irritancy as more organ-specific cells (Kemp *et al.*, 1983b; Shopsis and Sathe, 1984; Borenfreund and Borrero, 1984; Selling and Ekwall, 1985); this implies that the basal cytotoxic action of most toxicants is the critical feature in irritancy. It is probable that a battery of simple cell cultures could be substituted in place of the Draize test but a large-scale evaluation involving more classes of chemicals is required to establish this. Success in using culture methods as a replacement for the *in vivo* method must be attributed to comparable incubation times *in vitro* and *in vivo* and also to the direct application of the toxicant on the target tissue *in vivo*, thereby avoiding distortion of *in vitro* results due to deficient absorption or local pharmacokinetics. Corresponding research to evaluate dermal irritation by use of tissue cultures is not so advanced (Kao *et al.*, 1983), neither can eye irritancy be predicted by skin irritancy of chemicals (Williams, 1984).

7.5.3 Inhalable pollutants

Screening techniques to detect local toxicity have been used to investigate the effects of various substances on the lung. These include tobacco smoke (Chamson *et al.*, 1982; Curvall *et al.*, 1984), diesel exhausts (Zamora *et al.*, 1983; Lundborg *et al.*, 1983), and various dusts and fibres (Garrett *et al.*, 1981). One study (Brown and Poole, 1983) compared 21 mineral dusts in four different cell systems from various laboratories, and a similarity between toxicity to different cells, such as macrophages, V79-4 Chinese hamster fibroblasts, and erythrocytes was demonstrated. Comparison of the *in vitro* toxicity with the *in vivo* pathogenicity was hampered by sparsity of *in vivo* data. In another study (Curvall *et al.*, 1984), the toxicity of more than 300 tobacco chemicals was tested in four different cell systems; good agreement was obtained between systems and it was noted that biological activity could be related to chemical structure for many of the chemicals tested. It is unfortunate that these authors did not attempt to compare their results with the *in vivo* activity of the same compounds; by the use of test substances with a known and varied human lung toxicity, the same efforts would have resulted in more valuable information about the reliability of the methods.

7.5.4 Systemic toxicity

Several workers have attempted to predict acute systemic toxicity in man using various cellular systems (Allred *et al.*, 1982; Rodgers *et al.*, 1983; Johnson and Knowles, 1983; Wenzel and Cosma, 1983; Walum and Peterson, 1984; Reinhardt *et al.*, 1982). In many instances, relatively few substances have been tested by the new

methods and the experimental protocols did not provide for evaluation of the method by examination of the correlation between *in vitro* and *in vivo* toxicity. However, Walum and Peterson (1984) tested 35 common chemicals for their toxicity to neuroblastoma C1300 cells using a cell detachment method. For 30 of these substances, a positive correlation was found between cytotoxicity and the oral LD50 in rats; a very poor correlation was noted for five substances and this was attributed, in part, to the limited metabolic capacity of neuroblastoma cells. The authors concluded that batteries of cell tests are necessary to compensate for inaccurate predictions that might occur based on the results of a single test. Reinhardt *et al.* (1982) tested seven substances for toxicity in baby hamster kidney cells, as measured by cell detachment and cloning efficiency; both assays ranked all compounds tested according to their systemic *in vivo* toxicity. The relatively close relation between *in vitro* cytotoxicity and high (lethal) systemic toxicity found in these two studies confirms earlier studies (Lindgren *et al.*, 1983; Ekwall, 1983a,b) and is probably due to a very high frequency with which common chemicals exert a basal cytotoxic action at lethal doses for animals and man (Ekwall, 1983b). Thus, it would appear that screening procedures based on measurement of cytotoxicity in simple cell cultures can be used to detect chemicals that have acute systemic toxicity in man. Only a few substances exhibiting organizational toxicity, or which need metabolic activation for expression of their toxicity, or which otherwise have an organ-selective cytotoxicity will not be detected. This is different from the overall picture with short-term carcinogenicity tests, in which the group of chemicals requiring metabolic activation to express their carcinogenicity is fairly large. However, groups of chemicals, selected for specific biological activities, such as drugs and pesticides, are likely to include a higher percentage of members with an organizational toxicity than other groups so that the methods are likely to be less reliable in these cases.

A recent development is the use of rat hepatocytes for screening purposes (Cantilena *et al.*, 1983; Gray *et al.*, 1983; Story *et al.*, 1983; Tyson *et al.*, 1983). Story *et al.* (1983) tested 34 chemicals for cytotoxicity in rat hepatocytes, as measured by transaminase leakage after two and five hours of incubation. Good correlation between hepatotoxicity in an *in vitro* system and hepatotoxic responses *in vivo* was found. In a study of five haloalkanes, Tyson *et al.* (1983) showed that isolated hepatocyte systems have value for ranking structurally-related chemicals as to their cytotoxicity, even though their mechanisms of action may differ. For most compounds, the hepatocyte system probably measures basal cytotoxicity. Only studies of the apparent differences in toxicity between hepatocytes and undifferentiated cells *in vitro* (Ekwall and Acosta, 1982) or, more specifically, *in vitro/in vivo* comparisons can demonstrate the frequency of occurrence of organ-selective hepatotoxicity. One difficulty in interpreting the results of studies using hepatocytes is the time necessary for specific metabolite-mediated hepatotoxicity to be expressed clinically, compared with the relatively short incubation time used in the above discussed studies.

Testicular cell cultures (mixed cultures of Sertoli and germ cells) have recently been prepared and their response to some model testicular toxins studied (Gray and Beamand, 1984). Although this culture system has proved useful for limited series of congeners suspected to cause the testicular injury, its more general applicability remains unknown. In screening compounds with unknown toxicity, the use of such specific systems will not be cost-efficient since very few positives will result; at the same time, they are also laborious to carry out.

The most effective strategy for using tissue cultures for toxicity screening is to develop batteries of simple cell tests that measure basal cytotoxicity. It is probable that this can already be done with confidence for screening for eye irritant properties of chemicals. The battery should include hepatocytes, to estimate aspects of metabolically-induced toxicity, and possibly other systems of important organ-specific cells, too, if it is to be used to predict acute systemic toxicity. In addition to their use as screening tests basal cytotoxic and organ-selective cell tests can be used as analytical tools in conjunction with conventional animal toxicity tests to provide additional information on potential cytotoxicity and on mechanisms of toxic action.

7.6 *IN VITRO* CYTOTOXICITY AS A CRITERION FOR SELECTING 'PRIORITY CHEMICALS'

Chemical inventories, available in several countries including the US, Japan and EEC countries, indicate that a very large number of chemicals is being produced and marketed in many countries. For instance, about 55 000 commercial substances are listed in the US EPA TSCA inventory. Although about 9.5 per cent of the total number of substances reported (i.e. about 5000 chemicals) account for 99.9 per cent of the total production (Blair and Bowman, 1983) it is evident that exposure to relative low volume chemicals could be a major health problem for specific population groups under certain circumstances.

Toxicological data are currently either inadequate or non-existent for most existing chemicals (Grossblatt *et al.*, 1984; Silano *et al.*, 1986). In the absence of data concerning the health-related parameters, *in vitro* cytotoxicity data, which can be produced in a relatively short time and at a low relative cost, could be very useful for ranking chemicals with a similar exposure potential according to their potential toxicity and, possibly, to confirm other predictions based on consideration of their chemical structures. *In vitro* cytotoxicity data could also indicate the need for specific kinds of additional toxicity tests that would be required. Since industrial chemicals are not primarily of interest because of their specific biological activities, *in vitro* cytotoxicity data may provide a more useful criterion for priority ranking these chemicals than for other groups of chemicals such as drugs or pesticides. The use of a battery of tests with different cell types would be more appropriate than a single test in carrying out these screening tests. An established cell line, human diploid fibroblasts and rat hepatocytes could be used to provide tests with little variation, normal basic functions and metabolic capacity, respectively (see also Section 7.5).

Another possible approach is that used in the FRAME Research Programme on *in vitro* cytotoxicology (Balls and Bridges, 1983). FRAME has established a multicentre research programme in partnership with a number of industrial companies, to determine whether cell cultures can be used reliably in place of live animal methods currently used in routine acute toxicity tests. Two parallel thrusts are involved: (1) a co-ordinated attempt to devise a simple, rapid and inexpensive method for evaluating the gross toxic effects of chemicals on the fundamental properties of cultured cells; and (2) an attempt to increase the range of methods available to study the effects of chemicals on differentiated tissue-type specific properties of cells from common target organs. A human embryo lung fibroblast-like cell strain (BCL-D1) was used to detect the 'gross' toxic effects of chemicals on the 'fundamental' properties of cells, whereas eight additional cell types were selected for the study of the effects of chemicals on the specific properties of 'differentiated' cells. A xenobiotic metabolizing system was included in the *in vitro* cytotoxicity test protocol (Balls and Bridges, 1983).

7.7 FUTURE DEVELOPMENTS

There have been a number of important meetings in recent years that have discussed the use of tissue cultures in toxicology (Lindgren *et al.*, 1983; Williams *et al.*, 1983; Goldberg and Liebert, 1983; Balls *et al.*, 1983). These include 4 biannual European conferences on the use of tissue cultures in toxicology from 1980 to 1988 (published in *Toxicology and Xenobiotica*), and the annual conferences on methods and strategies for general toxicity testing with cell systems, arranged by the Scandinavian Society for Cell Toxicology, from 1983 to 1989 (published in *ATLA*). As time goes by, the research field thus begins to be structured by consensus among researchers. As mentioned in Section 7.4, three areas of research are now fairly well separated; toxicity testing, studies of toxic effects and mechanisms, and lastly, studies of metabolism of compounds.

A common problem to all areas and types of cell systems is the lack of standardization of procedures. Now, almost all laboratories produce results that are difficult to compare because of variations in methodology. Such results do not contribute to the common pool of knowledge of cytotoxicity, nor can they be collectively used in large-scale validation studies (Ekwall, 1980; Paganuzzi-Stammati *et al.*, 1981).

Another problem is the lack of good evaluation studies, designed to prove the value of tissue cultures in testing procedures. Such studies must be planned to account for basal cytotoxicity and selective cytotoxicity versus organizational toxicity, and also be performed on a rather large scale with many classes of chemicals. All forms of toxicity to human beings, acute and chronic, direct and indirect, various types of local and systemic toxicity, must be separately validated by systematic *in vitro*/*in vivo* comparisons since the success of tissue culture methods in predicting one type of toxicity in humans does not imply a similar success in predicting another type.

A third obstacle to the greater use of cytotoxicity testing today is confusion about the best use of different cell types and end-points of cellular toxicity (Ekwall and Ekwall, 1988). Basal toxicity is often measured in elaborate, organ-specific culture systems, while undifferentiated cells are used to measure organ-selective or even organizational toxicity in man. Inappropriate specific end-points are sometimes used to measure the gross inflammatory capacity of chemicals, while growth parameters are used to measure injury.

Future development within the area of toxicity testing will progress on several lines:

(1) Greater standardization of the methodology; standards for cells, media, and end-points must be established. Once this is done, more researchers will use established and documented methods in their work.

(2) Extensive validation of methods. The programme of the English organization FRAME has already made some progress in this respect and another collaborative validation study has recently been launched within the teratogenicity field—a long list of well-known teratogens with good animal data has been developed to be used in validation of tissue culture teratogenicity tests (Smith *et al.*, 1983). The Scandinavian Society of Cell Toxicology is now organizing a multilaboratory study by which a large number of toxicants with well-defined human acute toxicity can be tested *in vitro* (the multi-center evaluation of *in vitro* cytotoxicity, MEIC) (Bernson *et al.*, 1987, Bondesson *et al.*, 1989; Ekwall *et al.*, 1989). Studies of this kind could lead to a more definite opinion on the utility of cell culture tests in toxicological investigations. The outcome of these validation studies could also elucidate the current confusion about which cells and culture methods to use for various kinds toxicity testing, and assist in the development of test batteries for screening purposes.

REFERENCES

Acosta, D., Anuforo, D., and Smith, R.V. (1978). The use of primary liver cell culture to study hepatotoxic agents. *Toxicol. Appl. Pharmacol.*, **45**, 262–3.

Albert, A. (1979). *Selective Toxicity. The Physico-chemical Basis of Therapy*, Chapman & Hall, London.

Alexander, P. (1969). Comparison of the mode of action by which some alkylating agents and ionizing radiations kill mammalian cells. *Ann. N.Y. Acad. Sci.*, **163**, 652–75.

Allred, L.E., Oldham, J.W., Milo, G.E., Kindig, O., and Capen, C.C. (1982). Multiparametric evaluation of the toxic responses of normal human cells treated *in vitro* with different classes of environmental toxicants. *J. Toxicol. Environ. Health*, **10**, 143–56.

Ambesi-Impiombato, F.S., Parks, L.A.M., and Coon, H.G. (1980). Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc. Nat. Acad. Sci., USA*, **77**, 3455–9.

Auricchio, S., De Ritis, G., De Vincenzi, M., and Silano, V. (1985). Toxicity mechanisms of wheat and other cereals in celiac disease and related enteropathies. *J. Pediatr. Gastroenterol Nutr.*, **4**, 923–30.

Autian, J. (1977). Toxicological evaluation of biomaterials: Primary acute toxicity screening programme. *Artific. Organs*, **1**, 53–60.

Baird, W.M., Chemerys, R., Grinspan, J.B., Mueller, S.N., and Levine, E.M. (1980). Benzo(a)pyrene metabolism in bovine aortic endothelial and bovine lung fibroblast-like cell culture. *Cancer Res.*, **40**, 1781–6.

Balls M. and Bridges, J.W. (1983). The FRAME Research Programme on *in vitro* cytotoxicology. In: Goldberg, A.M., and Liebert, M.A. (Eds), *Alternative Methods in Toxicology*, volume 2, New York.

Balls, M., Ridell, R.J., and Worden, A. (Eds) (1983). *Animals and Alternatives in Toxicity Testing*, Academic Press, London.

Barnes, D., and Sato, G. (1980a). Methods for growth of cultured cells in serum-free medium. *Anal. Biochem.*, **102**, 255–70.

Barnes, D., and Sato, G. (1980b). Serum-free cell culture: a unifying approach. *Cell*, **22**, 649–55.

Barnes, D., Van der Bosch, J., Masui, H., Miyazaki, K., and Sato, G. (1980). The culture of human tumor cells in serum-free medium. In: Perstka, S. (Ed.), *Interferons, Methods in Enzymology*, volume 79. Academic Press, New York.

Bartsch, H., and Tomatis, L. (1983). Comparison between carcinogenicity and mutagenicity based on chemicals evaluated in the IARC monographs. *Environ. Health Perspect.*, **47**, 305–17.

Begue, J.M., Le Bigot, J.F., Guguen-Guillouzo, C., Kiechel, J.R., and Guillouzo, A. (1983). Cultured human adult hepatocytes: A new model for drug metabolism studies. *Biochem. Pharmacol.*, **32**, 1643–6.

Bernson, V., Bondesson, I., Ekwall, B., Stenberg, K., and Walum, E. (1987). A multicentre evaluation study of in vitro cytotoxicity *ATLA*, **14**, 144–6.

Bianchi, V. (1982). Nucleotide pool unbalance induced in cultured cells by treatments with different chemicals. *Toxicol.*, **25**, 13–18.

Bianchi, V., Debetto, P., Zantedeschi, A., and Levis, A.G. (1982). Effects of hexavalent chromium on the adenylate pool of hamster fibroblasts.*Toxicol.*, **25**, 19–30.

Bitensky, L. (1963). The reversible activation of lysosomes in normal cells and the effects of pathological conditions. In: de Renk, A.U.S., and Cameron, M.F. (Eds), *Lysosomes*, Churchill, London, pp. 362–75.

Blair, E.H., and Bowman, C.M. (1983). Control of existing chemicals. *ACS Symposium*, 67–79.

Bondesson, I., Ekwall, B., Hellberg, S., Romert, L., Stenberg, K., and Walum, E. (1989). MEIC—A new international multicenter project to evaluate the relevance to human toxicity of in vitro cytotoxicity tests. *Cell Biol. Toxicol.*, **5**, 331–47.

Borenfreund, E., and Borrero, O. (1984). *In vitro* cytotoxicity assays: Potential alternatives to the Draize ocular irritancy test. *Cell Biol. Toxicol.*, **1**, 33–9.

Brown, R.C., and Poole, A. (1983). The *in vitro* effects of mineral dusts. *ATLA*, **11**, 79–86.

Browne, R.M., and Tyas, M.J. (1979). Biological testing of dental restorative materials *in vitro*—A review. *J. Oral Rehabil.*, **6**, 365–74.

Burstein, S., Hunter, S.A., and Olzman, H. (1983). Prostaglandins and Cannabis. XII. The effects of cannabinoid structures on the synthesis of prostaglandins by human lung fibroblast. *Mol. Pharmacol.*, **23**, 121–6.

Burton, A.B.G., York, M., and Lawrence, R.S. (1981). The *in vitro* assessment of severe eye irritants. *Fd. Cosmet. Toxicol.*, **19**, 471–80.

Cantilena, L.R. Jr., Stacey, N.H., and Klassen, C.D. (1983). Isolated rat hepatocytes as a model system for screening chelators for use in cadmium intoxication. *Toxicol. Appl. Pharmacol.*, **67**, 257–63.

Castranova, V., Bowman, L., Reasor, M.J., and Miles, P.R. (1980). Effects of heavy metal ions on selected oxidative metabolic processes in rat alveolar macrophages. *Toxicol. Appl. Pharmacol.*, **53**, 14–23.

Chamson, A., Frey, J., and Hivert, M. (1982). Effects of tobacco smoke extracts on collagen biosynthesis by fibroblast cell cultures. *J. Toxicol. Environ. Health,* **9**, 921–32.

Costa, M. (1979). Levels of ornithine decarboxylase activation used as a simple marker of metal induced growth arrest in tissue culture. *Life Sci.*, **24**, 705–14.

Curvall, M., Enzell, C.R., and Pettersson, B. (1984). An evaluation of the utility of four *in vitro* short-term tests for predicting the cytotoxicity of individual compounds derived from tobacco smoke. *Cell Biol. Toxicol.*, **1**, 173–93.

Dolfini, E., Martini, A., Donelli, M.G., Morasca, L., and Garattini, S. (1973). Method for tissue culture evaluation of the cytotoxic activity of drugs active through the formation of metabolites. *Eur. J. Cancer,* **9**, 375–8.

Drake, S., Burns, R.L., and Nelson, J.A. (1982). Metabolism and mechanisms of action of 9-(tetrahydro-2-furyl)-6-mercaptopurine in chinese hamster cells. *Chem. Biol. Interact.*, **41**, 105–16.

Ekwall, B. (1980). Screening of toxic compounds in tissue culture. *Toxicol.*, **17**, 127–42.

Ekwall, B. (1983a). Correlation between cytoxocity *in vitro* and LD50 values. In: Lindgren, P., Thelestam, M., and Lindquist, N.G. (Eds), *First CFN Symposium on LD50 and Possible Alternatives. Acta Pharmacol. Toxicol.*, **52**, Suppl. 2, 80–99.

Ekwall, B. (1983b). Screening of toxic compounds in mammalian cell cultures. *Ann. N.Y. Acad. Sci.*, **407**, 64–77.

Ekwall, B., and Acosta, D. (1982). *In vitro* comparative toxicity of selected drugs and chemicals in HeLa cells, Chang liver cells and rat hepatocytes. *Drug Chem. Toxicol.*, **5**, 219–31.

Ekwall, B., Bondesson, I., Castell, J.V., Gómez-Lechón, M.J., Hellberg, S., Högberg, J., Jover, R., Ponsoda, X., Romert, L., Stenberg, K., and Walum, E. (1989). Cytotoxicity evaluation for the first ten MEIC chemicals: Acute lethal toxicity in man predicted by cytotoxicity in five cellular assays and by oral LD50 in rodents. *ATLA*, **17**, 83–100.

Ekwall, B., and Ekwall, K. (1988). Comments on the use of diverse cell systems in toxicity testing. *ATLA*, **15**, 193–200.

Elferink, J.G.R. (1979). Chlorpromazine inhibits phagocytosis and exocytosis in rabbit polymorphonuclear leukocytes. *Biochem. Pharmacol.*, **28**, 965–8.

Engvall, E., Oshima, R.G., Brennan, M.J. and Ruoslahti, E. (1984). Clonal tumorigenic endodermal cell lines producing basement membrane components. *Exp. Cell Res.*, **150**, 258–67.

Fedoroff, S. (1966). Proposed usage of animal tissue culture terms. *In vitro*, **2**, 155–9.

Fedoroff, S. (1977). Primary cultures, cell lines and cell strains: Terminology and characteristics. In: Fedoroff, S., and Hertz, L. (Eds), *Cell, Tissue and Organ Cultures in Neurobiology,* Academic Press, New York, San Francisco and London, pp. 265–86.

Garrett, N.E., Campbell, J.A., Stack, H.F., Waters, M.D., and Lewtas, J. (1981). The utilization of the rabbit alveolar macrophage and chinese hamster ovary cell for evaluation of the toxicity of particulate materials. I. Model compounds and metal-coated fly ash. *Environ. Res.*, **24**, 345–65.

Goldberg, A.M., and Liebert, M. (1983). Alternative methods in toxicology. In: *Product Safety Evaluation*, New York.

Gray, T.J.B., and Beamand, J.A. (1984). Effect of some phthalate esters and other testicular toxins on primary cultures of testicular cells. *Fd. Chem. Toxicol.*, **22**, 123–31.

Gray, T.J.B., Lake, B.G., Beamand, J.A., Foster, J.R., and Gangolli, S.D. (1983). Peroxisome proliferation in primary cultures of rat hepatocytes. *Toxicol. Appl. Pharmacol.*, **67**, 15–25.

Grisham, J.W. (1979). Use of hepatic cell cultures to detect and evaluate the mechanisms of action of toxic chemicals. *Int. Rev. Exp. Pathol.*, **20**, 123–210.

Grossblatt, N., Whittenberger, J.L., Bailar, J.C., Doull, J., Pfitzer, E.A., and Upton, A.C. (1984). Toxicity testing—Strategies to determine needs and priorities. National Academy Press, Washington, D.C.

Guess, W.L., Rosenbluth, S.A., Schmidt, B., and Autian, J. (1965). Agar diffusion method for toxicity screening of plastics on cultured cell monolayers. *J. Pharm. Sci.*, **54**, 1545–7.

Guguen-Guillouzo, C., Gripon, P., Vandenberghe, Y., Lamballe, F., Ratanasavanh, D., and Guillouzo A. (1988). Hepatotoxicity and molecular aspects of hepatocyte function in primary culture. *Xenobiot.*, **18**, 773–83.

Guillouzo, A., Le Bigot, J.F., Guguen-Guillouzo, C., and Kiechel, J.R. (1982). Presence of Phase I and Phase II drug metabolizing enzymes in cultured human foetal hepatocytes. *Biochem. Pharmacol.*, **31**, 2427–9.

Harmon, H.J., and Sanborn, M.R. (1982). Effect of naphthalene on respiration in heart mitochondria and intact cultured cells. *Environ. Res.*, **29**, 160–73.

Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell. Res.*, **25**, 585–621.

Hensten-Pettersen, A., and Helgeland, K. (1977). Evaluation of biologic effects of dental materials using four different cell culture techniques. *Scand. J. Dent. Res.*, **85**, 291–6.

Hensten-Pettersen, A., and Helgeland, K. (1981). Sensitivity of different human cell lines in the biologic evaluation of dental resin-based restorative materials. *Scand. J. Dent. Res.*, **89**, 102–7.

Higuchi, K. (1976). Cultivation of mammalian cell lines in serum free chemically defined medium. *Methods Cell Biol.*, **14**, 131–43.

Hoidal, J.R., Fox, R.B., Le Marbe, P.A., Perri, R., and Repine, J.E. (1981). Altered oxidative metabolic response *in vitro* of alveolar macrophages from asymptomatic cigarette smokers. *Am. Rev. Resp. Dis.*, **123**, 85–9.

Holden, H.T., Lichter, W., and Siegel, M.M. (1973). Quantitative methods for measuring cell growth and death. In: Kruse, P. Jr., and Patterson, M.K. Jr. (Eds), *Tissue Culture: Methods and Applications*, Academic Press, New York, pp. 408–11.

Hucker, H.B. (1970). Species differences in drug metabolism. *Ann. Rev. Pharm.*, **10**, 99–118.

Johnson, T.L., and Knowles, C.O. (1983). Effects of organotins on red platelets. *Toxicol.*, **29**, 39–48.

Kao, J., Hall, J., and Holland, J.M. (1983). Quantitation of cutaneous toxicity: An *in vitro* approach using skin organ culture. *Toxicol. Appl. Pharmacol.*, **68**, 206–17.

Kasten, F.H., Felder, S.M., Gettleman, L., and Alchediak, T. (1982). A model culture system with human gingival fibroblasts for evaluating the cytotoxicity of dental materials. *In Vitro*, **18**, 650–60.

Kemp, R.B., Meredith, R.W.J., Gamble, S., and Frost, M. (1983a). A rapid cell culture technique for assessing the toxicity of detergent-based products *in vitro* as a possible screen for eye irritancy *in vivo*. *Cytobios*, **36**, 153–9.

Kemp, R.B., Meredith, R.W.J., Gamble, S., and Frost, M. (1983b). Toxicity of detergent-based commercial products on cells of a mouse line in suspension culture as a possible screen for the Draize rabbit eye irritation test. *ATLA*, **11**, 15–21.

Klaassen, C.D., and Stacey, N.H. (1982). Use of isolated hepatocytes in toxicity assessment. In: Plaa, G., and Hewitt, W.R. (Eds), *Toxicology of the Liver*, Raven Press, New York, pp. 147–79.

Krejci, L. (1976). Effect of eye drugs on corneal epithelium. Comparative test on tissue cultures. *Cesk. Oftalmol.*, **32**, 163–8.

Lamb, R.G., and Schwartz, D.W. (1982). The effects of bromobenzene and carbon tetrachloride exposure *in vitro* on phospholipase C activity in rat liver cells. *Toxicol. Appl. Pharmacol.*, **63**, 216–29.

Lieberman, M., Adam, W.J., and Bullock, P.N. (1980). The cultured heart cell: problems and prospects. In: Harris, C.C., Trumpand, B.F., and Stoner, G.D. (Eds), *Methods in Cell Biol.*, Academic Press, New York, p. 187.

Lindgren, P., Thelestam, M., and Lindquist, N.G. (Eds) (1983). First CFN Symposium on LD50 and possible alternatives. *Acta Pharmacol. Toxicol.*, **52**, Suppl. II.

Lundborg, P., Cederberg, I., Wiklund, L., and Petterson, B. (1983). Effects of waste incineration combustion emissions measured by some toxicity test systems. *Toxicol. Lett.*, **19**, 97–107.

Miller, J.A., and Miller, E.C. (1971). Chemical carcinogenesis: Mechanisms and approaches to its control. *J. Nat. Cancer Inst.*, **47**, V–XIV.

Moldeus, T., Jones, D.P., Ormstad, K., and Orrenius, S. (1978). Formation and metabolism of a gluthatione-S-conjugate in isolated rat liver and kidney cells. *Biochem. Biophys. Res. Commun.*, **83**, 195–200.

Muir, C.K. (1983). The toxic effects of some industrial chemicals on rabbit ileum *in vitro* compared with eye irritancy *in vivo*. *Toxicol. Lett.*, **19**, 309–12.

Muir, C.K. (1984). Further investigations on the ileum model as a possible alternative to *in vivo* eye irritancy testing. *ATLA*, **1**, 129–34.

Murakami, H., and Masui, H. (1980). Hormonal control of human colon carcinoma cell growth in serum-free medium. *Proc. Nat. Acad. Sci. ISA*, **77**, 3464–8.

Nakada, S., Saito, H., and Imura, N. (1981). Effects of methylmercury and inorganic mercury on the nerve growth factor-induced neurite outgrowth in chick embryonic sensory ganglia. *Toxicol. Lett.*, **6**. 23–28.

Nardone, R.M. (1977). Toxicity testing *in vitro*. In: Rothblat, G.H., and Cristofalo, J. (Eds), *Growth, Nutrition and Metabolism of Cell in Culture*, volume III, Academic Press, New York, San Francisco, London, pp. 471–95.

Nardone, R.M. (1983). Neurotoxicity testing: An *in vitro* strategy. *Ann. N.Y. Acad. Sci.*, **407**, 458–9.

North-Root, H., Yackovich, F., Demetrulias, J., Gacula, M. Jr., and Heinze, J.E. (1982). Evaluation of an *in vitro* cell toxicity test using rabbit corneal cells to predict the eye irritation potential of surfactants. *Toxicol. Lett.*, **14**, 207–12.

Orly, J., Sato, G., and Erickson, G. (1980). Serum suppresses the expression of hormonally-induced functions in cultured granulosa cells. *Cell*, **20**, 817–27.

Paganuzzi-Stammati, A., Silano, V., and Zucco, F. (1981). Toxicology investigations with cell culture systems. *Toxicol.*, **20**, 91–153.

Quinn, G.P., Axelrod, J., and Brodie, B.B. (1958). Species, strain and sex differences in metabolism of hexobarbitone, amidopyrine, antipyrine and aniline. *Biochem. Pharmacol.*, **1**, 152–9.

Reinhardt, C.A., Schawalder, H., and Zbinden, G. (1982). Cell detachment and cloning efficiency as parameters for cytotoxicity. *Toxicol.*, **25**, 47–52.

Rodgers, C.G., Héroux-Metcalf, C., and Iverson, F. (1983). *In vitro* cytotoxicity of polychlorinated biphenyls (Aochlors 1016, 1242, 1254 and 1260) and their effect on phospholipid and neutral lipid composition of Chinese hamster ovary (CHO-K1) cells. *Toxicol.*, **26**, 113–24.

Sato, H.G., and Yasamura, Y. (1966). Retention of differentiated function in dispersed cell culture. *Trans. N.Y. Acad. Sci.*, **28**, 1063–79.

Sato, G.H., Pardee, A.B., and Sirbasku, D.A. (Eds) (1982). Growth of cells in hormonally defined medium. Cold Spring Harbor Conference on cell proliferation, Vol. 9, Book A and B, Cold Spring Harbor Laboratory.

Scaife, M.C. (1983). *In vitro* studies on ocular irritancy. In Balls, M., Ridell, R.G., and Worden, A. (Eds), *Animals and Alternatives in Toxicity Testing*, Academic Press, London, pp. 367–71.

Schaeffer, W.I. (1984). Usage of vertebrate, invertebrate and plant cell, tissue and organ culture terminology. *In Vitro*, **20**(1): 19–24.

Schiller, C.M., and Lucier, G.W. (1978). The differential response of isolated intestinal crypt and tip cells to the inductive action of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Chem. Biol. Interact.*, **22**, 199–209.

Schwartz, H.S., and Mihic, E. (1973). Species and tissue differences in drug selectivity. In: Mihic, E. (Ed.), *Drug Resistance and Selectivity. Biochemical and Cellular Basis*, Academic Press, News York, pp. 413–49.

Selling, J., and Ekwall, B. (1985). Screening for eye irritancy using cultured HeLa cells. *Xenobiot.*, **15**, 713–17.

Shopsis, C., and Sathe, S. (1984). Uridine uptake inhibition as a cytotoxicity test: correlations with the Draize test. *Toxicol.*, **29**, 195–206.

Silano, V., Stammati-Paganuzzi, A., and Vittozzi, L. (1986). Possibilities for in vitro methods. In: *Chemicals Testing and Animal Welfare, Proceedings*, Swedish National Chemicals Inspectorate, Stockholm. pp. 152–182.

Smith, M.K., Kimmel, G.L., Kochhar, D.M., Shepard, T.H., Spielberg, S.P. and Wilson, J.G. (Eds) (1983). A selection of candidate compounds for *in vitro* teratogenesis test validation. *Teratog. Carcinog. Mutagen.*, **3**, 461–80.

Spangberg, L. (1973). Kinetic quantitative evaluation of material cytotoxicity *in vitro*. *Oral Surg.*, **35**, 389.

Stacey, N.H., and Klaassen, C.D. (1981a). Comparison of the effects of metals on cellular injury and lipid peroxidation in isolated rat hepatocytes. *J. Toxicol. Environ. Health*, **7**, 139–47.

Stacey, N.H., and Klaassen, C.D. (1981b). Inhibition of lipid peroxidation without prevention of cellular injury in isolated hepatocytes. *Toxicol. Appl. Pharmacol.*, **58**, 8–18.

Stacey, N.H., Ottenwalder, H., and Kappas, H. (1982). CCl_4-induced lipid peroxidation in isolated rat hepatocytes with different oxygen concentration. *Toxicol. Appl. Phrmacol.*, **62**, 421–7.

Stanford, J.W. (Ed.) (1980). Recommended standard practices for biological evaluation of dental materials. *Int. Dent., J.*, **30**, 140–88.

Story, D.L., Gee, S.J., Tyson, C.A., and Gould, D.H. (1983). Response of isolated hepatocytes to organic and inorganic cytotoxins. *J. Toxicol. Environ. Health*, **11**, 483–501.

Striker, G.E., Harlan, J.M., and Schwartz, S.M. (1980). Human endothelial cells *in vitro*. In: Harris, C.C., Trump, B.F., and Stoner, G.C. (Eds), *Methods in Cell Biology*, Academic Press, New York, London, Toronto, Sydney, San Francisco, p. 135.

Swanston, D.W. (1983). Eye irritancy testing. In: Balls, M., Ridell, R.J., and Worden, A. (Eds), *Animals and Alternatives in Toxicity Testing*, Academic Press, London, pp. 337–65.

Tell, R.W., and Douglas, W.H.J. (1980). Aryl hydrocarbon hydroxylase activity in Type II alveolar lung cells. *Experimentia*, **36**, 107.

Tyson, C.A., Hawk Prather, K., Story, D.L., and Gould, D.H. (1983). Correlations of *in vitro* and *in vivo* hepatotoxicity for five haloalkanes. *Toxicol. Appl. Pharmacol.*, **70**, 289–302.

Wahlberg, J.A. (1983). Exfoliative cytology as a refinement of the Draize eye irritancy test. *Toxicol. Lett.*, **18**, 49–55.

Walton, J.R. (1975). The systemic appraisal of cellular injury. *Agents and Actions*, **5**, 394–400.

Walton, J.R., and Buckley, I.K. (1975). Cell models in the study of mechanisms of toxicity. *Agents and Actions*, **5**, 69–88.

Walum, E., and Peterson, A. (1984). Acute toxic action of chemicals in cultures of mouse neuroblastoma C 1300 cells. *J. Toxicol. Environ. Health*, **13**, 511–20.

Waters, M.D., Vaughan, T.O., Abernethy, D.J., Garland, H.R., Cox, C.C., and Coffin, D.L. (1975). Toxicity of platinum (IV) salts for cells of pulmonary origin. *Environ. Health Perspect.*, **12**, 45–56.

Weisburger, H.H., Grantham, P.H., and Weisburger, E.R. (1964). Metabolism of N-2-fluorenylacetamide in the hamster. *Toxicol. Appl. Pharmacol.*, **6**, 427–33.

Wennberg, A., Hasselgren, G., and Tronstad, L. (1979). A method for toxicity screening of biomaterials using cells cultured on millipore filters. *J. Biomed. Mater. Res.*, **13**, 109–20.

Wenzel, D.G., and Cosma, G.N. (1983). A quantitative metabolic inhibition test for screening toxic compounds with cultured cells. *Toxicol.*, **29**, 173–82.

White, F.V., Ceccarini, C., Georgieff, I., Matthieu, J.-M., and Costantino-Ceccarini, E. (1983). Growth properties and biochemical characterization of mouse Schwann cells cultured *in vitro*. *Exp. Cell Res.*, **148**, 183–94.

Williams, A.J., and Cole, P.J. (1981). *In vitro* stimulation of alveolar macrophage metabolic activity by polystyrene in the absence of phagocytosis. *Br. J. Exp. Pathol.*, **62**, 1–7.

Williams, G.M., Dunkel, V.C., and Ray, V.A. (Eds) (1983). Cellular systems for toxicity testing. *Ann. N.Y. Acad. Sci.*, p. 407.

Williams, S.J. (1984). Prediction of ocular irritancy potential from dermal irritation test results. *Fd. Chem. Toxicol.*, **22**, 157–61.

Williams, S.J., Graepel, G.J., and Kennedy, G.L. (1982). Evaluation of ocular irritancy potential: interlaboratory variability and effect of dosage volume. *Toxicol. Lett.*, **12**, 235–41.

York, M., Lawrence, R.S., and Gibson, G.B. (1982). An *in vitro* test for the assessment of eye irritancy in consumer products—Preliminary findings. *Int. J. Cosmet. Sci.*, 223–34.

Yoshida, M., Onaka, M., Fujita, T., and Nakajima, M. (1979). Inhibitory effects of pesticides on growth and respiration of cultured cells. *Pesticide Biochem. Physiol.*, **10**, 313–21.

Zamora, P.O., Benson, J.M., Marshall, T.C., Mokler, B.V., Li, A.P., Dahl, A.R., Brooks, A.L., and McClellan, R.O. (1983). Cytotoxicity and mutagenicity of vapor-phase pollutants in rat lung epithelial cells and chinese hamster ovary cells grown on collagen gels. *J. Toxicol. Environ. Health*, **12**, 27–38.