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CHAPTER 6

*Conceptual Approaches to Methodology Development**

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6.1 INTRODUCTION

There is a societal need and responsibility for public authorities to attempt to ensure that individuals in society are protected from harm. Given the current state of our knowledge, this necessitates some testing in animals and extrapolation of the results, no matter how difficult, to predict likely human responses. We are still far too ignorant to predict the toxic effects of a compound from first principles. Therefore, we have to fall back on appropriate models of the human system to identify possible hazards. By necessity, this leads to the use of mammals or other species that often respond in a sufficiently similar manner to humans to provide an index of the potential hazard.

Nevertheless, the problems of extrapolation and evaluation are formidable. Thousands of new chemicals need to be evaluated every year while only a fraction of the estimated 65 000 chemicals (Maugh, 1978) in use today have been subject to testing according to available public information (National Research Council, 1984). In addition, the present animal testing techniques are generally crude, cumbersome and costly and there is growing public criticism of such use of animals.

Among toxicologists, there are some who see animal testing as an unsatisfactory answer to toxicology's problems. Thus, in 1971, Rofe, in an excellent but little-cited review of the use of tissue culture in toxicology, stated that:

> In seeking to bridge the gap between the effects of foreign substances on animals and their effects on man, it seems unlikely that a substantial contribution to the problem can be made by prolonging the conventional toxicological procedures or including additional organ function tests.

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Others, scientific and non-scientific, have also criticized animal testing (Rofe, 1971; Melmon, 1976; Muul *et al.*, 1976; Zbinden, 1976; Heywood, 1978; Stevenson, 1979; Efron, 1984), commenting variously that toxicology has sometimes created more problems than it has solved in the last decade, that toxicology is a science without scientific underpinning, and that we should move towards the development of an appropriate battery of short-term tests, using both *in vitro* and *in vivo* approaches, to assess product safety.

The techniques most commonly highlighted as having potential for the future are (i) cell and organ culture, (ii) computer modelling, and (iii) the use of less invasive animal procedures and endpoints producing little stress or suffering. There have already been a number of reviews of the cell and organ culture in toxicology investigations beginning with a seminal paper by Pomerat and Leake in 1954, followed by a number of reviews after 1970 (Rofe, 1971; Dawson, 1972; Worden, 1974; Nardone, 1977; Berky and Sherrod, 1978; Tardiff, 1978; Deutsche Pharmakologische Gesellschaft, 1980; Stammati *et al.*, 1981; Zucco and Hooisma, 1982; Ekwall, 1983; Grisham and Smith, 1984). However, judging from the number of citations to these papers, none have had impact. According to the records of the *Science Citation Index*, Rofe's 1971 review has been cited less than twenty times in the following twelve years with a high of three citations each in 1976 and 1980. Although more and more toxicological research is being conducted *in vitro*, the potential of cell culture as applied to toxicological evaluation and hazard assessment is only now beginning to be tested and assessed. This is the result of public pressure, the availability of funds for such studies, and increased concern among scientists.

However, the use of cell cultures in toxicology testing and hazard assessment must be developed and implemented cautiously. Obviously, a single cell culture cannot accurately mimic the complex interactions of all the cell types in the body no matter how exquisite the experimental design. *In vivo* metabolism may be simulated to some extent but not completely (Fry and Bridges, 1977) and other integrating functions (e.g. hormones, immune reactions, phagocytosis) are not included. In addition, a cell culture is a relatively static system in which the dose of the test chemical reaching the target system and the duration of contact may not be the same as those that occur in the *in vivo* test. There are also physical problems regarding solubility, stability and biophysical effects of the test compound.

On the other hand, the technique of cell culture has great potential once investigators have acquired the background knowledge to ask highly focused and specific questions. The static nature of cell culture is also an advantage in that the dose and duration of contact of a test chemical can be precisely determined. Much less test chemical is required to carry out cell culture investigations than to conduct tests *in vivo*. Replicate cultures can be set up with ease and generate more data in a short time.

One of the most exciting aspects of cell culture studies in toxicology is that one can use human tissue. Such studies have been limited in the past because of the

difficulty of maintaining and growing differentiated human cell types in culture but the technical problems are being steadily overcome. For example, important developments in the last years include improvements in the quality control of media and plasticware provided by manufacturers, improved quality control in the laboratory, better media formulations (Barnes and Sato, 1980) for the growth of normal cells as well as for cells exhibiting specialized functions (e.g. heart cell contractility and melanin production by melanocytes), and improvements in cell separation and cloning techniques (cf. Nardone and Bradlaw, 1983).

Nardone and Bradlaw (1983) describe four interfaces between *in vitro* methodology and animal toxicology—screening tests, mechanistic studies, personnel monitoring and considerations for risk assessment. They note that screening tests are the most developed and are likely to remain the major focus of *in vitro* toxicology. However, mechanistic studies probably will become steadily more important, both in toxicological evaluations and for risk assessment. One could also classify *in vitro* methodology according to whether the approach is empirical, model development or mechanistic (Goldberg, 1984a).

The empirical approach for the development of methodology is problematic. The questions asked are generally not focused and the intent is to develop correlations prior to fundamental understandings. Additionally, the results tend to be somewhat unpredictable. Should this be the case in the development of *in vitro* toxicological methods, we will have, unfortunately, provided supplementary testing strategies but not replacement testing strategies. This will leave us with the dilemma of attempting to use the *in vitro* methodologies without being able to rely on them.

Model development attempts to utilize systems that mimic the *in vivo* systems. Generally, the model system is neither complete nor faithful in all aspects of the system being modelled, but it tends to provide useful information if the data are not over-interpreted. In those model systems where a single aspect of an integrated response is examined and the data are interpreted, it can provide meaningful inferences for the evaluation of chemical effects.

The mechanistic approach in the development of *in vitro* methodologies should be based on a thorough knowledge of the underlying metabolism, kinetics and biology in the system or species to be examined. If the metabolic pathways are understood, or if it is known that the parent compound produces the toxicological insult, then one can develop a system to examine the mechanisms by which the chemical(s) works. That is, one can examine the adverse chemical or physical effects that lead to a significant functional loss in the tissue or system. This approach allows the *in vitro* system to be derived from the species under study. It also provides a better understanding of the chemical–biological interaction and the consequences of that interaction. Once a mechanism has been identified, it may then be possible to develop appropriate, interpretable, simple and reliable *in vitro* methodologies.

From a scientific viewpoint, the mechanistic approach is not only preferable but necessary. *In vitro* methods will be more acceptable and will develop rapidly

when the knowledge base has advanced far enough to permit a focus on mechanisms.

6.2 ALTERNATIVES IN TOXICOLOGY

Toxicity testing on animals may be divided into acute, subacute and chronic tests. Acute tests are those in which the animals are dosed with one or a few doses of the test compound and kept for at most a few weeks. Such tests include protocols for determining the various LD50s as well as eye and skin irritancy tests. Up to 50 per cent of all animals used in toxicology testing are killed in acute tests (Rowan, 1984). Subchronic tests last from a few weeks to several months. Chronic tests last for more than three months and include tests for reproductive and carcinogenic effects among others. The search for new approaches in all these areas will continue to evolve. However, at the present time, our lack of knowledge about the mechanisms of possible toxic insults is such that some animal testing is going to be required.

6.2.1 Acute toxicity testing

In acute tests, the investigator is observing an immediate response in which the orgnaism's defence mechanisms are rapidly overwhelmed. Where specific end-points are being determined (e.g. eye irritancy) it may well be possible to develop an adequate *in vitro* alternative based on one or more screening systems. However, one of the functions of acute testing is the identification of unexpected toxic effects. The empiricism of this approach requires that a relatively good model for the whole human being be used. This generally means using a whole mammal because the metabolism and response of other mammals is at least sufficiently similar to human responses to provide an index of hazard. However, there are acute tests for which the prospect of either reducing the number of animals used, or for developing an adequate *in vitro* test are relatively good and these are discussed below.

6.2.1.1 LD50 testing

The calculation of the median lethal dose (LD50) for the measurement of toxicity was introduced in 1927 (Trevan, 1927). At that time, determination of the LD50 was used to standardize such potent biologicals as digitalis, insulin and diptheria toxin. With time, however, the LD50 came to be used as a standard measure by which the toxicity of all chemicals was assessed. In 1968, Morrison, Quinton and Reinert questioned this use of the LD50 (Morrison *et al.*, 1968), arguing that the classical test used too many animals and that the statistical figure resulting was quite meaningless. They contended that a figure, generated from the use of 6–10 animals, was the best that could be achieved given the inadequacies of the test system (Hunter *et al.*, 1978). There have been several recent criticisms of the LD50 (Zbinden and Flury-Roversi, 1981; Rowan, 1983; Goldberg, 1984b). As a result of scientific

criticism, coupled with political pressure from the animal welfare movement, the classical LD50 test (with a few specific exceptions) appears to be on its way out as a regulatory requirement. For example, the German authorities state that they are accepting acute toxicity test data using a small number of animals (Bass *et al.*, 1982; Veberla and Schnieders, 1982) and the Food and Drug Administration has now explicitly stated that it has no requirement for LD50 tests and that acute toxicity data from alternative tests may well be acceptable (Food and Drug Administration, 1984).

The alternatives that are being considered all require the use of far fewer animals. Bruce (1985) has proposed the use of six to ten animals in the Up–Down method (Dixon and Mood, 1948) although it cannot be recommended for testing materials where delayed deaths (more than a few days) are the rule. Several simplifications of the standard method, all of which require fewer animals, have recently been proposed (Muller and Kley, 1982; Schutz and Fuchs, 1982; Tattersall, 1982; Lorke, 1983) and the last (Lorke, 1983), which recommends the use of only thirteen animals, is claimed to be suitable for industrial use where a variety of chemicals of widely differing toxicities must be assessed. Where only an estimate is required, the method proposed by Deichmann and LeBlanc offers yet another choice (Deichmann and LeBlanc, 1943).

Another approach which has also been suggested is the use of a structure–activity computer model to estimate LD50s (Enslein and Craig, 1978; Enslein *et al.*, 1983). This approach has been criticized because the chemicals used to design the models were not congeneric and because the biological end-point (death) used is not the function of a single active site in a well-defined system (Rekker, 1980). The developers of the model argue that there is no question that the use of a congeneric set of chemicals would produce tighter estimates but that this is insufficient reason not to explore a model based on heterogeneous collections of chemicals. As this field of quantitative structure–toxicity relationships (QSTR) develops, one can anticipate major strides in the use of these systems as predictors of toxicity (Golberg, 1983).

There have been several papers which have correlated the results of cytotoxicity assays with animal LD50s (Barile and Hardegree, 1970; Sako, 1977; Autian and Dillingham, 1978; Ekwall, 1980; Balls and Bridges, 1984) but the development of an adequate cell culture alternative is very unlikely. There are many different toxic effects, and a crude cytotoxicity assay is unlikely to be successful as a general screen for acute toxicity. In addition, these non-mechanistic tests may result in the identification of an excessive number of false-positives and false-negatives and efforts to correlate cytotoxicity data with questionable LD50 figures are unlikely to yield significant toxicological insights. Nevertheless, there is a clear need for good cytotoxicity data and for the development of reliable measures of cytotoxicity (cf. Autian and Dillingham, 1978; Balls and Bridges, 1984).

Therefore, the present state of development of alternative approaches to the classical LD50 test is focused on the use of fewer animals (up to a 90 per cent reduction) with more attention being paid to morbidity and symptoms than a

statistical estimate of the median lethal dose. For most purposes, the use of small numbers of animals to estimate the median lethal dose appears to be a satisfactory alternative. Cell culture systems have been investigated but they cannot provide the breadth of coverage of possible toxic insults of a simple *in vivo* mammalian organism. A computer model for estimating LD50s has been developed (Enslein *et al.*, 1983). While it allows one to estimate the toxicity of a new substance quickly, the computer model suffers limitations as a possible replacement to the animal test.

6.2.1.2 Ophthalmic irritancy testing

The classic method for assessing the potential for ophthalmic irritancy of chemicals is the Draize eye irritancy test (Draize *et al.*, 1944; Freidenwald *et al.*, 1944). In recent years, this test has been criticized by both scientists (Weil and Scala, 1971; Griffith *et al.*, 1980) and by animal welfare groups (Rowan, 1981). In fact, in 1978, Smyth commented that the Draize eye irritancy test was one area where a search for a non-animal alternative had a real chance of success (Smyth, 1978). A recent review of eye irritation testing outlines some of the difficulties in identifying eye irritants and the specific historical background of, and problems with the Draize Eye Irritancy test (Falahee *et al.*, 1981). For example, one of the main difficulties with this test as a regulatory tool is identified as the subjective nature of scoring and evaluating the test response.

Pressure from animal welfare campaigns has, in recent years, resulted in the support of a number of projects to seek an alternative to the Draize eye irritancy test with promising results. The projects can be divided into those investigating modifications of the test which would result in less animal distress, and those investigating *in vitro* and protozoan systems as possible replacements (cf. Nardone and Bradlaw, 1983).

(*a*) Refinements to the classical Draize eye irritancy test. The test modifications which have been proposed include the use of smaller volumes (Griffith *et al.*, 1980), which would reduce the severity of the reaction as well as permitting the investigator to develop dose–response curves, the use of local anaesthetics (Falahee *et al.*, 1981), an exfoliative cytology test which is reportedly more sensitive and more easily quantified than the classic Draize eye irritancy test (Walberg, 1983), and the identification of all severe dermal irritants as eye irritants without further testing. Griffith and his colleagues have argued, with some justification, that the use of a single 100 µl aliquot for eye irritation testing is inappropriate. They suggest that a 10 µl aliquot (and higher multiples) is retained in the eye better and that dose–response curves can be developed if necessary (Griffith *et al.*, 1980). In most cases, the use of smaller quantities of material being placed in the eye will result in less irritation and, therefore, less animal distress.

In recent years, there have been several investigations of the use of local anaesthetics in the eye during ophthalmic testing as a means of reducing animal suffering. Ulsamer *et al.* (1977) have reported that butacaine sulphate provided

adequate anaesthesia without notably affecting the irritancy scores. Hoheise (personal communication, 1984) indicates that a double dose of tetracaine (separated by 10 minutes) is more effective in abolishing pain and interferes less with the irritant response, although Walberg disputes this (Walberg, 1983). Johnson (1980) reports that amethocaine HCl is also effective. In a trial of 31 substances, the anaesthetic either had no effect, or produced an increase in the irritant response and did not, therefore, mask irritancy.

Walberg (1983) has developed a very promising modification to the Draize eye irritancy test which is less stressful to the animal, more sensitive and more easily quantified. The eye is exposed to the test substances and then, at standard intervals after the exposure, exfoliated cells are retrieved from the conjunctival sac via a distilled water rinse. The number of cells retrieved is a very sensitive index of irritancy and correlates well with published Draize eye irritancy test scores. The approach needs further validation but appears to be promising as a more sensitive and more objective approach to eye irritancy testing. The greater sensitivity of the exfoliative cytology test also means that smaller or more dilute doses of irritant substances could be used, thereby causing less trauma and distress.

It has also been suggested that a rapid and simple approach to the elimination of most severe irritants from eye testing, and to reduce the number of rabbits required, would be to pre-test materials for primary skin irritation or other properties. However, Williams (1984) reports that of 60 materials that were found to be severe primary skin irritants or corrosive to the skin and that had also been tested for primary eye irritancy, only 34 were also severe eye irritants. Fifteen of the 60 were only mildly irritating or non-irritants in the eye test. Williams cautions, therefore, that it may be misleading to classify a substance as an eye irritant solely on the basis of dermal irritancy. He suggests that the 24-hour occlusion method used in skin testing may well overwhelm physiological defence mechanisms. The lack of correlation between dermal and ophthalmic scores may be due to overestimation of the dermal response by current test procedures. With regard to other properties such as pH, substances with a pH of 12 or more are usually regarded as eye irritants. However, Murphy *et al.* (1982) cautions that there is no simple rule for predicting irritancy from the pH. Acetic acid (5 per cent), with a pH of 2.7, produces substantial corneal opacity while 0.3 per cent hydrochloric acid (pH of 1.3) causes no corneal opacity. At the other end of the scale, 2.5 per cent ammonium hydroxide (pH 11.8) produced corneal opacity while 0.3 per cent sodium hydroxide (pH 12.8) did not. Nevertheless, Walz (1984) reports a clear relation between irritation (oedematous reaction after intracutaneous injection) and pH in a mouse skin test of tissue compatible buffers. Buffers with a pH of below 3 and above 11.5 caused irritation. The boundary for the alkalis was very sharp.

(*b*) Replacement methods for the classical Draize eye irritancy test. A wide range of *in vitro* and protozoan systems have been proposed as possible alternatives (at least as preliminary screens) for the Draize eye irritancy test. Nardone and Bradlaw (1983) have already reviewed many of these including the use of the enucleated

eyes (rabbit), human or rabbit cornea cell cultures, other types of cell culture, and the chorioallantois of chick embryos. Some of the first attempts to devise a specific alternative to the Draize eye irritancy test were undertaken in Britain using mouse (Simons, 1980) or human buccal cavity mucosa cells (Bell *et al.*, 1979). The authors of both reports indicated that the *in vitro* approach showed promise but that much more work would be needed to develop and validate an adequate test system. While there have been a spate of recent research reports (Carter *et al.*, 1973; Burton *et al.*, 1981; McCormack, 1981; North-Root *et al.*, 1982; Scaife, 1982; Chan and Haschke, 1983; Douglas and Spilman, 1983; Leighton *et al.*, 1983; Muir *et al.*, 1983; Silverman, 1983; Borenfreund and Borrero, 1984; Muir, 1984; Shopsis and Sathe, 1984) from investigators seeking an alternative to the Draize eye irritancy test, there is still no clear indication of which approach, or approaches, might be the most effective.

Cytotoxicity and cell morphology studies appear to be the favoured approach but few of the studies have gone beyond a characterization of the *in vitro* system. Douglas and Spilman chose to develop a human ocular cell culture as an *in vitro* assay since it would retain species-specific and organ-specific characteristics (Douglas and Spilman, 1983). They chose corneal tissue since corneal damage is the most heavily weighted in scoring damage in the Draize eye irritancy test. They further required that the test system should be practical for routine use and that the assay be based on cell perturbations which are relevant to *in vivo* irritation (e.g. ^{51}Cr release, LDH release, uptake of A1B (a non-metabolized amino acid), and rhodamine uptake (as an index of mitochondrial function)). Although the preliminary results from ^{51}Cr release were promising, the project was, unfortunately, not completed.

While Douglas and others have favoured the idea of using corneal cells to match, as far as it is possible, organ-specific characteristics, Borenfreund and Borrero (1984) report that cells from different organs and species appear to give very similar results, indicating that it may not be that important to match cell culture type with the target organ. The results of Borenfreund's cytotoxicity and morphology assay indicate reasonable correlation with Draize eye irritancy test scores and also with another possible alternative based on a cellular uridine-transport assay developed in the same laboratory (Shopsis and Sathe, 1984).

Another approach has involved the use of whole enucleated rabbit (Burton *et al.*, 1981) or bovine (Carter *et al.*, 1973) eyes. Burton *et al.* (1981) report that the enucleated eyes remain viable for at least four hours and that there is good correlation of the results from this system (using a measurement of corneal swelling) with *in vivo* eye irritancy. However, although these whole eye systems may be useful as predictors of human eye irritation, Douglas and Spilman (1983) argue that such systems are poorly suited to the screening of a large number of compounds or of many replicate samples.

It has been suggested that cell culture systems are not well suited to predicting how fast the eye might recover from the toxic insult. However, Chan and Haschke (1983) are working with a corneal cell culture system which might be used to

predict recovery from injury and Jumblatt and Neufeldt (1983) have described a cell culture model for wound closure studies.

Two other *in vitro* models using the chick chorioallantoic membrane (Leighton *et al*, 1983) and excised guinea-pig ileum (Muir *et al*., 1983; Muir, 1984) have also been reported recently. Leighton *et al*., (1983) are developing the chorioallantoic membrane (CAM) from the chick embryo as a non-sentient but intact organ which could be used to evaluate irritation and inflammation. The initial reports are based on tests conducted with fairly strong acid and alkali solutions and measurement of the size of the resultant lesion. This is an end-point which requires refinement. There have also been problems from background irritation caused by shell fragments falling on the CAM when the aperture is cut. Nevertheless, the CAM system could be a very promising model for modelling inflammatory responses provided a simple but elegant end-point can be developed.

Many new model systems have been investigated in the past few years and some already show considerable promise as improvements on the Draize eye irritancy test or as the basis for rapid screening systems. However, at the present time, none of the *in vitro* systems have yet been sufficiently validated or evaluated to be considered as replacements to the classical or modified Draize eye irritancy test.

6.2.1.3 Dermal toxicity testing

Some of the same approaches applied to the search for alternatives to ophthalmic irritancy testing would probably be successful for dermal irritancy testing. There are difficulties in extrapolating from animal to humans, for example (Kligman 1982; Marks 1983). However, very little research into possible *in vitro* systems for identifying skin irritants has, so far, been undertaken. There have been isolated reports of the use of *in vitro* skin cultures to study toxic reactions or mechanisms (cf. Fouts, 1982; Imokawa and Okamoto, 1983; Kao *et al*., 1983) but there has been no concerted programme to seek an *in vitro* screening test for irritancy and cutaneous toxicity. Another area of dermal toxicity is phototoxicity which is now routinely evaluated in animals. Several alternative methods have been investigated (Weinberg and Springer, 1981; Morrison *et al*., 1982; McAuliffe *et al*., 1983; Tenenbaum *et al*., 1984) but are still at a relatively early stage of development. More information on dermal toxicity is presented elsewhere in this volume.

6.2.1.4 Other organs

One area of acute toxicity where alternative methods may be expected to contribute to our understanding of potential chemical insult concerns the acute reactions of isolated organs or cell cultures to large doses such as might occur during unintentional exposures. The setting of public emergency limits and the development of appropriate therapies for acute poisoning cases could find data derived from *in vitro*

organotypic systems to be invaluable. Little attention has been paid to this area of acute organ toxicity.

6.2.2 Chronic toxicity testing

In chronic toxicity testing where the investigator is assessing the likelihood for both targeted (e.g. carcinogenicity) and non-targeted (e.g. disorder in lipid metabolism) effects, we are much more likely to be able to predict human hazards if we understand the mechanism of the toxic insult than if we continue to rely on empirical testing approaches. In the acute toxicity field discussed above, there has been a focused, funded effort to find alternatives following both empirical and mechanistic lines. In chronic toxicity testing, a similar effort is underway to develop short-term tests to identify mutagens, carcinogens and teratogens, but not to investigate organ-specific effects. We will be discussing some of the issues in developing alternatives in chronic toxicity testing, specifically for hepatotoxicity, neurotoxicity and teratogenicity but will not discuss carcinogenicity and mutagenicity. Other chapters in this volume will discuss specific organ systems.

6.3 CONCLUSION

This brief review of alternative approaches to acute toxicity tests and eye irritancy testing provides an introduction to some new conceptual approaches to toxicology testing. A simple empirical search for *in vitro* tests that correlate with various toxic endpoints will not only be insufficient, it will be detrimental. The possibility of developing superior methods for safety evaluation is much more likely to be realized if mechanistic approaches are used when investigating *in vitro* tests. For cell cultures, both animal and human, to be used to their full potential, the culture techniques must be considerably improved. Fully defined growth media must be developed which will support the growth of a wide range of defined cells. It is now possible to maintain and grow many different types of cells which express differentiated function *in vitro*. For example, changing culture conditions allowed one group of investigators to establish a thyroid cell line which expressed differentiated thyroid cell characteristics even after three years of continuous culture (Ambesi-Impiombata *et al.*, 1980). Also, beating heart cells can be maintained for a week in good condition and have been used to investigate anaesthetic (Miletich *et al.*, 1983) and isoproterenol (Ramos *et al.*, 1983) cardiotoxicity.

Computer-assisted structure–activity relationships in toxicology have not yet been developed. As toxicology data bases and our understanding of mechanisms improve, so will the potential applicability of quantitative structure–toxicity relationships (Craig, 1983; Golberg, 1983; Wold *et al.*, 1983).

With the exciting advances now taking place in the disciplines that contribute to toxicology (e.g. molecular biology, cell biology), the time is opportune for academic, industrial and regulatory toxicologists to explore new avenues for safety

evaluation. This will mean discarding tests which no longer do what they are meant to and developing new ones which provide better assessments of potential human hazards.

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