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PERSPECTIVES ON ALTERNATIVES TO CURRENT ANIMAL TESTING TECHNIQUES IN PRECLINICAL TOXICOLOGY

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INTRODUCTION

In ancient times, knowledge of the biological properties of natural substances, both medicinal and lethal, was a vital source of power for witch doctors and magician priests. Today, given the steady increase in public concern about the possible deleterious effects of chemicals in our environment, the information the toxicologist possesses is once again an important influence in society. Toxicologists are being asked to provide answers to ever more complex questions. Unfortunately, the knowledge base and the techniques currently being used to acquire that knowledge are often unequal to the task.

In the early 1960s, both the thalidomide disaster (1) and the publication of *Silent Spring* by Rachel Carson (2) dramatized the possible negative impact of potent new pharmaceuticals and pesticides. As a result, government authorities throughout Europe, North America, and Japan responded to public concern by demanding a dramatic increase in the requirements for premarket testing of new products. Existing animal tests were refined and more animal tests were developed in an attempt to evaluate the safety of drugs, food additives,

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pesticides, and other chemicals. Between 1965 and 1979, one company reported that the costs of toxicology testing increased by a factor of 3.2, even after correcting for the effects of inflation (3). One factor contributing to these costs is the enormous numbers of animals now consumed in routine toxicology testing, accounting for an estimated 20% of the 50–70 million laboratory animals used every year in the United States (4).

Public authorities undoubtedly bear a responsibility 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 extrapolating the results, no matter how difficult, to predict likely human responses. We are still far too ignorant to know the toxic effects of a compound from first principles (via theoretical toxicology?). Therefore, we must 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 respond in a manner sufficiently similar to humans to provide an index of the potential hazard. Nevertheless, the problems of extrapolation and evaluation are formidable. Thousands of new chemicals must be investigated every year, while only a fraction of the estimated 65,000 chemicals (5) in common use have been subject to testing according to available public information (6). In addition, the present animal testing technologies are generally crude, cumbersome, and costly, and there is growing public criticism of animal use.

Among toxicologists, there are some who see animal testing as an unsatisfactory answer to toxicology's problems. In 1971, Rofe (7), in an excellent but little-cited review on the use of tissue culture in toxicology, stated: "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."

Others, scientific and non-scientific, have also criticized animal testing (7–13), commenting variously that in the last decade toxicology has sometimes created more problems than it has solved, that toxicology is a science without scientific underpinning, and that toxicologists should move toward the development of an appropriate battery of short-term tests, using both in vitro and in vivo approaches, to assess product safety.

Since 1975, criticism of animal testing from another source, the animal welfare movement, has grown considerably. For the most part, the bulk of this criticism has been targeted against the Draize eye irritancy (14) and the LD50 tests (15). The animal welfare campaigns have been effective. Campaigns have not only brought about a reevaluation of the rationale for Draize eye irritancy and LD50 testing, they have also spurred a reassessment of the entire animal-testing approach. In general, the animal activists advocate the elimination of the use of animals in toxicity testing and the establishment of non-animal

alternatives, in particular tissue culture and computer modeling. The most effective campaigners recognize that these are long-term goals and in the short term seek to reduce animal use as much as is feasible by eliminating unnecessary animal tests or developing, validating, and using potential alternatives as fast as possible.

The combination of these two forces, internal toxicological self-criticism and external animal welfare pressure, has begun to have an impact. Numerous workshops, plenary sessions, and full-scale symposia have been organized in the last three to four years to discuss testing on animals and the development of alternatives (cf. 16–20). Over four million dollars from private sources has been allocated to support research on potential new approaches, and government authorities also are beginning to recognize the appropriateness and necessity for in vitro methods of toxicological evaluation (21).

Given these changes, it is important that the concept of alternatives be clearly explained and that the potential for developing and using appropriate alternatives be carefully considered. This review attempts to introduce the reader to the concept of alternatives and its implications in toxicology testing. Recent relevant research advances will also be briefly discussed. The authors have catalogued alternative approaches to the subject rather than attempting a thorough critical evaluation. The use of animals and alternatives in mutagenicity and carcinogenicity testing will not be covered in any detail. There is already an extensive literature on in vitro tests for carcinogenicity and mutagenicity, and the reader is referred to several recent reviews (22–25) for discussion of these topics.

WHAT ARE ALTERNATIVES?

The term *alternatives* is a relatively recent addition to the campaign literature of the animal-protection movement, and there is still widespread disagreement over its precise definition. Some organizations use the term to refer only to techniques that replace completely the use of animals in a particular area, for example, a computer model to predict LD50 values. However, others follow the definition developed by Russell & Burch in 1959 (26); they defined an alternative as any technique that replaces the use of animals, that reduces the need for animals in a particular test, or that refines a technique in order to reduce the amount of suffering endured by the animal. Thus, use of the up-down method to determine an acute toxicity value (27) is an alternative to the classical LD50 test because fewer animals are required. These three R's represent the common definition of alternatives; we define the term this way in this article.

One of the key features of the concept of alternatives is that it refers to

techniques used in biomedical research. Thus, a discussion of alternatives in toxicology testing is really only a review of the merits of different methodological approaches with a particular focus on ways to reduce animal use and suffering. The alternatives most commonly considered are cell and organ culture, computer modeling, and the use of minimally invasive procedures and endpoints that produce little stress or suffering. A number of reviews of the use of cell and organ culture in toxicology investigations have already been published, beginning with a seminal paper by Pomerat & Leake in 1954 (28), followed by a number of reviews after 1970 (7, 29–38). However, judging from the number of citations to these papers, none has 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 in toxicological evaluation and hazard assessment is only now beginning to be tested and evaluated. 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 must be developed and implemented cautiously in toxicology testing and hazard assessment. Obviously, a single cell culture cannot mimic the complex interactions of all cell types in the body, no matter how exquisite the experimental design. In vivo metabolism may be simulated to some extent but not completely (39), and integrating functions such as hormones, immune reactions, and phagocytosis cannot be duplicated. 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. Cell cultures also present physical problems regarding the solubility, stability, and biophysical effects of the test compound.

On the other hand, cell culture technique 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. Far less of the test chemical is required for cell culture investigations than in in vivo tests. Therefore, one can easily set up replicate cultures 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 growing and maintaining differentiated human cell types in culture. But technical problems are being steadily overcome. Important developments in the last years include improvements in the quality control of media and the plasticware provided by manufacturers, improved quality control in the laboratory, better media formulations (40) 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. 41).

Nardone & Bradlaw (41) describe four interfaces between in vitro methodology and animal toxicology: screening tests, mechanistic studies, personnel monitoring, and considerations for risk assessment. Screening tests are the most developed and are likely to remain the major focus of in vitro toxicology. However, mechanistic studies probably will become increasingly 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 (42).

The empirical approach to the development of methodology is problematic. The questions asked are generally not focused and correlations develop 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 unfortunately have 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 utilizes systems that try to mimic in vivo systems. Generally, the model system is neither complete nor faithful in all aspects of the system being modeled, but it tends to provide useful information if the data are not overinterpreted. In those model systems where a single aspect of an integrated response is examined and the data are interpreted in that single system, this technique can provide meaningful inferences for the evaluation of chemical effects.

The mechanistic approach to the development of in vitro methodologies should be based on a thorough knowledge of the metabolism, kinetics, and biology of 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 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.

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 various LD50's as well as eye and skin irritancy tests. Up to 50% of all animals used in toxicology testing are killed in acute tests (4). 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 alternatives 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.

Acute Toxicity Testing

In acute tests, the investigator observes an immediate response in which the organism's defense mechanisms are rapidly overwhelmed. Where specific endpoints are being determined (e.g. eye irritancy), it may 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 is relatively good. These are discussed below.

LD50 TESTING The calculation of median lethal dose (LD50) for the measurement of toxicity was introduced in 1927 (43). At that time, determination of the LD50 was used to standardize such potent biologicals as digitalis, insulin, and diphtheria 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 (44), arguing that the classical test used too many animals and that the statistical figure resulting was meaningless. They contended that a figure generated from the use of six to ten animals was the best that could be achieved given the inadequacies of the test system (45). More recently, several others have also criticized the LD50 approach (46–48). 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 require-

ment. For example, the German authorities have said that they will accept acute toxicity test data using small numbers of animals (49, 50), and the Food and Drug Administration has explicitly stated that it no longer requires LD50 tests and that acute toxicity data from alternative tests may be acceptable (51).

Alternative tests for acute toxicity all require far fewer animals than the traditional methods. Bruce (27) has proposed the use of six to ten animals in the up-down method (52), although this technique 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 been proposed (53–56), and the most recent (56), 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 & LeBlanc offers yet another choice (57).

Another suggested approach is the use of a structure-activity computer model to estimate LD50's (58, 59). This approach has been criticized because the chemicals used to design the models were not congeneric and because the biological endpoint (death) used is not the function of a single active site in a well-defined system (60). 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 heterogenous 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 predicters of toxicity (61).

Several papers have correlated the results of cytotoxicity assays with animal LD50's (62–66), 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 nonmechanistic 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, good cytotoxicity data and the development of reliable measures of cytotoxicity (cf. 63, 66) are clearly needed.

The present state of alternatives development for the classical LD50 test is focused on the use of fewer animals (up to a 90% reduction), with more attention being paid to morbidity and symptoms than to 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 LD50's has been developed (58). While it allows one to estimate the toxicity of a new substance quickly, it suffers limitations as a possible replacement for animal tests.

OPHTHALMIC IRRITANCY TESTING The classic method for assessing the potential for the ophthalmic irritancy of chemicals is the Draize eye irritancy test (67, 68). In recent years, this test has been criticized by both scientists (69, 70) and by animal welfare groups (71). In fact, in 1978, Smyth commented that the Draize eye irritancy test is one area where a search for a non-animal alternative has a real chance of success (72). A recent review of eye irritation testing outlines some of the difficulties in identifying eye irritants, as well as the specific historical background of and problems with the Draize eye irritancy test (73). For example, one of the main difficulties with this test as a regulatory tool is the subjective nature of scoring and evaluating the response.

Pressure from animal welfare campaigns in recent years has led to support for a number of projects seeking an alternative to the Draize eye irritancy test, with promising results. Some attempt to modify the test to reduce animal distress, and others are investigating in vitro and protozoan systems as possible replacements for it (cf. 41).

Refinements to the classical Draize eye irritancy test Proposed test modifications to the Draize eye irritancy test include the use of smaller volumes (70), which would reduce the severity of the reaction as well as permit the development of dose-response curves; the use of local anesthetics (73); the use of an exfoliative cytology test, which is reportedly more sensitive and more easily quantified than the classic test (74); 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 (70). In most cases, the use of smaller quantities of material 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 anesthetics in the eye during ophthalmic testing as a means of reducing animal suffering. Ulsamer has reported that butacaine sulfate provides adequate anesthesia without notably affecting the irritancy scores (75). Hoheisel (personal communication) indicates that two doses of tetracaine (separated by ten minutes) are more effective in abolishing pain and interfere less with the irritant response, although Walberg disputes this (74). Johnson reports that amethocaine HCl is also effective. In a trial of thirty-one substances, the anesthetic either had no effect or produced an increase in the irritant response and did not therefore mask irritancy (76).

Walberg has developed a very promising modification to the Draize eye irritancy test that is less stressful to the animal, more sensitive, and more easily quantified than the classic test (74). The eye is exposed to the test substances; then at standard intervals after 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 it appears to be a more sensitive and more objective approach to eye irritancy testing. The greater sensitivity of the exfoliative cytology test means that smaller or more dilute doses of irritant substances can be used, thereby causing less trauma and distress.

Some researchers have suggested that a simple and rapid approach to the elimination of most severe eye irritant tests that would also reduce the number of rabbits required is to pretest materials for primary skin irritation or other properties. However, Williams (77) investigated sixty materials for primary eye irritancy that were also severe primary skin irritants or corrosive to the skin. Of these, only thirty-four were severe eye irritants; fifteen of the sixty were only mildly irritating or were not irritating at all. 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 twenty-four hour occlusion method used in skin testing may well overwhelm physiological defense mechanisms. The lack of correlation between dermal and ophthalmic scores may be due to an overestimation of the dermal response by current test procedures.

Substances with a pH of 12 or more are usually regarded as eye irritants. However, Murphy and colleagues (78) caution that there is no simple rule for predicting irritancy from pH. Acetic acid (5%) with a pH of 2.7 produces substantial corneal opacity, while 0.3% hydrochloric acid (pH 1.3) causes no corneal opacity. At the other end of the scale, 2.5% ammonium hydroxide (pH 11.8) produces corneal opacity, while 0.3% sodium hydroxide (pH 12.8) does not. Nevertheless, Walz (79) reports a clear relation between irritation (edematous 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.

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 & Bradlaw (41) have already reviewed many of these systems, including those using enucleated eyes of rabbit, human or rabbit corneal 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 (80) or human buccal mucosa cells (81). The authors of both reports indicate that the in vitro approach shows promise but that much more work is needed to develop and validate an adequate test system. While there have been a spate of recent research reports (82–94) from investigators seeking an alternative to the Draize eye irritancy test, there is still no clear indication of which approach or approaches will be the most effective.

Cytotoxicity and cell morphology studies appear to be the favored approach in this regard, but few of them have gone beyond a characterization of the in vitro system. Douglas & Spilman chose to develop a human ocular cell culture as an in vitro assay, since it retains species-specific and organ-specific characteristics (91). They chose corneal tissue since corneal damage is the most heavily weighted in the scores of the Draize eye irritancy test. They further required that the test system be practical for routine use and that the assay be based on cell perturbations relevant to in vivo irritation [e.g. ^{51}Cr release, LDH release, uptake of AIB (a nonmetabolized 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 favored the idea of using corneal cells to match, as far as is possible, organ-specific characteristics, Borenfreund (87) reports 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 as well as with another possible alternative based on a cellular uridine-transport assay developed in the same laboratory (86).

Another approach has involved the use of whole enucleated rabbit (85) or bovine (93) eyes. Burton et al (85) report that 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 whole eye systems may be useful as predictors of human eye irritation, Douglas argues that they are poorly suited to the screening of a large number of compounds or of many replicate samples (91).

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 (83) is working with a corneal cell culture system that might predict recovery from injury and Jumblatt & Neufeldt (95) have described a cell culture model for wound closure studies.

Two other in vitro models using the chick chorioallantoic membrane (88) and excised guinea pig ileum (89, 90) have also been reported recently. Leighton is developing the chorioallantoic membrane (CAM) from the chick embryo as a nonsentient but intact organ that can be used to evaluate irritation and inflammation (88). The initial reports are based on tests conducted with

fairly strong acid and alkali solutions that measure the size of the resultant lesion. This is an endpoint that requires refinement. In addition, background irritation caused by shell fragments falling on the CAM when the aperture is cut has also caused problems. Nevertheless, the CAM system could be a very promising method for modeling inflammatory responses provided a simple but elegant endpoint 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 has yet been sufficiently validated or evaluated to be considered as replacements to the classical or modified Draize test.

DERMAL TOXICITY TESTING As mentioned above, Williams's (77) analysis of the skin and eye irritancy of a range of substances indicates that severe skin irritancy does not reliably predict severe eye irritancy. In discussing this result, he raises the possibility that the twenty-four hour occlusion used in the standard skin test may be too severe. For example, better correlation was found between skin and eye irritation when only a four-hour occlusion period was used in the skin test. The Organization for Economic Cooperation and Development (OECD) guidelines also call for only a four-hour occlusion. This is not the only question that has been raised about skin irritancy tests. Marks (96) notes that there are many differences between the skin of common test animals (rats, guinea pigs, rabbits, and mice) and human skin. Kligman (97) points out that the dose-response relationship for irritants is flat in animal skin and as a result discriminative ability is low. Furthermore, animal testing appears to be of little value for detecting mild human skin irritants. Marks (96) suggests that one way to overcome this difficulty is to test substances of low and moderate toxicity in human volunteers (with suitable safeguards), although he recognizes that such testing may be limited by ethical and regulatory restrictions.

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. 98–100), but no concerted program to seek an in vitro screening test for irritancy and cutaneous toxicity has been carried out. Part of the problem is the lack of a reliable supply of human skin samples, and it is probably desirable to use human material rather than animal tissues in these tests. However, adequate supplies of rudimentary human epidermal sheets may become available for toxicity studies as a result of work being done to develop an artificial epidermal substitute for burn victims (101, 102). In addition, some of the irritancy test systems now being developed as alternatives to the Draize test may also be suitable for testing possible skin irritancy. For example, if the problems with the chick

chorioallantoic membrane system (88) can be resolved, it could become a useful system for identifying inflammatory responses. Another approach to the assessment of dermal absorption in animals may be the use of a lipid-impregnated filter (103).

Phototoxicity is now routinely evaluated in animals, but the methods are time-consuming, expensive, and not always good predictors of the human response. Several publications evaluate possible in vitro assays for phototoxicity. For example, a number of authors have used yeast growth inhibition (cf. 104, 105) and have reported good correlation between this inhibition and acute, nonphotoallergic phototoxicity. Another recent approach has employed human peripheral blood monocytes, with inhibition of mitogen-stimulated thymidine incorporation as an endpoint (106, 107). Investigators using this approach argue that their test avoids the problem of false-negative results and note that preliminary data from a human lymphoblastoid cell line indicate that these cells perform well in the assay (106). Use of human lymphoblastoid cells would simplify the problem of cell supply and standardization if the test is found to be sufficiently reliable to use as a standard screening system.

The investigation of in vitro tests for dermal toxicity has not progressed as far as that for ophthalmic toxicity tests. Nevertheless, the principles in each case are the same, and we are therefore optimistic about the potential for developing a dermal irritancy alternative.

OTHER ORGANS One area of acute toxicity where alternative methods may contribute to our understanding of potential chemical insult is the acute reactions of isolated organs or cell cultures to large doses such as might occur during unintentional exposure. The setting of public emergency limits and the development of appropriate therapies for acute poisoning cases could find data from in vitro organotypic systems to be invaluable. Little attention has been paid to this area of acute organ toxicity. Some in vitro work relevant to these issues is discussed in the section on chronic toxicity below.

Chronic Toxicity Testing

In chronic toxicity testing that assesses the likelihood of both targeted (e.g. carcinogenicity) and nontargeted (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 that follow 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 discuss some of the issues in developing alternatives in chronic toxicity testing, specifically for hepatotoxic-

ity, neurotoxicity, and teratogenicity. We will not discuss carcinogenicity and mutagenicity.

HEPATOTOXICITY The liver is particularly vulnerable to injury by ingested chemicals because it receives higher concentrations of chemicals absorbed from the intestine than do other organs and it plays a major role in the biotransformation, concentration, and excretion of xenobiotics or their metabolites. The liver is therefore an important target organ in any evaluation of the toxic potential of a chemical. Liver function is generally assessed by hepatic excretion measurements and chemical and histological analyses (108). The development of a simple in vitro test system to evaluate hepatotoxicity would be very useful.

Several in vitro approaches for the evaluation of hepatotoxicity have already been investigated, including perfused liver (109), liver cell suspensions (110), and various types of liver cell cultures (110–113). However, all these systems have disadvantages that limit their usefulness (112). Perfused liver preparations are viable for only a few hours, are technically complicated, and show limited reproducibility from one laboratory to another. Liver cell lines lose many of the differentiated functions of normal hepatocytes in vitro. Freshly prepared hepatocyte suspensions usually demonstrate cell damage and impaired enzyme functions, while primary hepatocyte cultures usually contain only a fraction of in vivo levels of microsomal drug-metabolizing enzymes such as cytochrome P-450. However, several investigators have recently demonstrated that one can maintain good levels of cytochrome P-450 by manipulating the culture medium (114–116). Omitting cystine (and cysteine) and adding 5-aminolevulinic acid and nicotinamide (or metarypone) to the usual cell culture media maintains cytochrome P-450 at levels close to in vivo ones for up to seven days (112, 115).

Liver cell cultures show considerable promise for the investigation of the mechanism of action of hepatotoxins and, as culture methods are improved and differentiated liver cell functions are maintained longer in vitro (117), the research and testing potential of such systems will expand. Hepatocyte cultures have been used successfully in detailed toxicological studies in relatively few laboratories around the world (cf. 111, 112), and then they have been used to study mechanisms of toxicity. Only limited attention has been given to their potential usefulness in routine screening tests.

NEUROTOXICITY Although the development of in vitro systems to investigate hepatotoxicity may appear a daunting task, the development of systems for the routine investigation of neurotoxicity in vitro is likely to be even more difficult. The nervous system is extremely complex and it is composed of many different cell types. Many neurotoxins affect only one specific cell type in the nervous system, and the full range of potential neurotoxic effects cannot be

evaluated in any single in vitro system. Nevertheless, some in vitro and invertebrate test systems have potential as screens for specific neurotoxins (118, 119).

Damstra & Bondy (120) discuss the usefulness of neurochemical approaches, in vivo and in vitro, in neurotoxicological studies. However, they caution the unwary investigator of the many pitfalls of such tests. For example, changes in body temperature, such as those caused by amphetamine or chlorpromazine, may affect neurochemistry, while food deprivation causes an increased turnover of 5-hydroxytryptamine as a result of increases in free-serum tryptophan. Nevertheless, recent advances in neurochemistry permit analysis of an ever-increasing range of specific processes in nerve cells affected by neurotoxins.

Another approach to the development of rapid and reliable neurotoxicity screens is the use of invertebrates whose relatively simple nervous systems still possess sufficient complexity to be of use. Best (121) argues that fresh-water planaria fit these requirements and that they could prove to be very useful in neurotoxicological studies.

The culture of nervous system tissues of various types has now become commonplace, ranging from organotypic cultures through primary cell cultures to the cultures of the various tumor cell lines such as neuroblastomas, gliomas, and pheochromocytomas. Schrier (122) discusses the advantages and disadvantages of various cell and organ cultures in neurotoxicology studies and emphasizes that the greatest potential for cell culture systems is in investigations of mechanisms. Nevertheless, some believe that cell cultures can be used as general screening systems. For example, Fedalei & Nardone (123) report that they have developed a neuroblastoma (N1E-115) assay for organophosphate toxicity that might be used as an in vitro alternative to the hen brain assay for neurotoxic esterase, the usual test for organophosphate toxicity. Nardone is also investigating the development of a neuroblastoma screening test for acrylamides (124).

Despite these developments, Dewar, in a comprehensive review of neurotoxicity studies (125), emphasizes that in all probability no single technique will ever be able to detect all possible neurotoxic endpoints. Nevertheless, he urges that more effort be put into the development of in vitro biochemical tests for certain well-defined neurotoxic endpoints. He also argues that nervous cell cultures should be further investigated and developed and that efforts to validate lower vertebrate models, such as Xenopus tadpoles, as neurotoxicological screens should be encouraged.

FETOTOXICITY After the thalidomide disaster, more stringent requirements for animal tests of fetal toxicity were established. However, the routine animal test systems have not proved particularly reliable predictors of hazards to the

human fetus. For example, the human fetus is fifty times more sensitive to thalidomide than the rabbit but is totally insensitive to corticosteroid-induced cleft palate, to which rabbits and rodents are very susceptible. The standard animal tests using rodents or rabbits are not only relatively poor predictors of human hazard, they are also expensive and time-consuming. As a result, a number of investigators have attempted to develop an in vitro screening test for teratogenicity.

One of the problems with the search for a satisfactory in vitro screen is that a number of different mechanisms appear to lead to one of a range of different outcomes, all of which result in some feature of fetotoxicity (e.g. living or dead terata, resorption or spontaneous abortion of embryos, functional impairment of offspring, or underweight offspring). Wilson (126) suggests that certain key aspects of reproductive success need to be tested, including mutagenesis, epigenesis, and organogenesis. He also describes the essential criteria for an ideal in vitro teratogenicity screen; it must be a system that uses large numbers of subjects, that is relevant to mechanisms of teratogenesis, that is easy to use, and that yields uniform and repeatable responses. Wilson (126) favors the use of fish embryos, Drosophila larva, sea urchin embryos, or chick embryos, although he cautions that only the chick embryo has been sufficiently investigated to offer promise for early validation as a preliminary screen for teratogenicity.

In the last few years, a number of other systems have been proposed as possible screening tests for teratogenicity. The available in vitro systems may be broadly classified into the following categories.

Mammalian embryo or embryo organ culture The maintenance and development in culture of whole rodent embryos from day 9.5 to day 12.5 has been described and developed by New (127), who suggests using the system for screening teratogens (128). Several groups have begun to employ the technique for this purpose (129, 130, 132), and one group (131) has cultured the embryos in human serum to identify the possible teratogenicity of drugs being taken by human subjects. This system could be very useful as a screen as it is further refined and developed.

Use of other veterbrate embryos The most studied of the other vertebrate embryos is the chick embryo, which has been extensively investigated as a possible teratogen screen (cf. 133, 134). However, the chick embryo is very sensitive to a wide range of experimental and chemical treatments, and this reduced discrimination may limit its usefulness for a single-purpose test. On the other hand, recent modifications to the shell-windowing procedure (135) may help reduce the embryo's sensitivity to experimental manipulations and thus render it more useful.

Birge and co-workers (136) have proposed that fish and amphibians in the embryo-larval stages constitute simple and effective models to investigate teratogenesis and to screen for environmental compounds that may be of concern to human health. They report large differences in sensitivity to test chemicals among six different amphibians. For example, bullfrog larva have an LC50 for atrazine of 0.41 mg/l, while larva of the American toad have an LC50 greater than 48 mg/l. Dumont & Schultz (137) also promote an amphibian system using Xenopus for screening environmental mixtures.

Invertebrate systems Several invertebrate systems have been proposed as suitable for teratogen screening, including Drosophila (138), the cricket *(Acheta domesticus)* (139), and hydra (140). The Drosophila test has been more extensively investigated than the cricket system. In a recent trial using Drosophila (the system detects interference with muscle and/or neuron differentiation in embryo cell cultures), researchers reported that the test correctly identified all but six of the one hundred chemicals investigated (138). The authors also researched strain differences and dose-responses of a few selected chemicals and suggested that the system might be useful in studying teratogenic mechanisms. Johnson & Gabel (140), however, argue that one cannot classify substances as being teratogenic or non-teratogenic, since the terms have little meaning in terms of hazard to the conceptus. It is more important to establish whether there is a large difference between the dose that produces toxicity in the adult (A) and the dose that affects development (D). Where the A/D ratio is close to unity, the test substance has no specific developmental toxicity, but when the ratio is large (for thalidomide the ratio is 60), this indicates substantial risk to the conceptus.

Johnson & Gabel (140) propose that an artificial embryo system composed of dissociated and pelleted hydra cells be used to study the effects on development and that the adult hydra be used to study general toxicity. They report agreement between the A/D ratios for mammals compared to the ratios for their hydra system.

Cell culture systems Several investigators have proposed using differentiating cells (141–144, 147) or non-differentiating cells (145, 146) in culture as a teratogen screen. They variously suggest that cell cultures could be useful screening systems because they assess sensitivity to cell-cell interactions (146), cell killing and reduction in cell proliferation (142, 145), and disruption of cellular anabolism involved in morphogenesis and organogenesis (141, 143–144). Some of these systems (142, 146) are currently being validated in a trial sponsored by the National Toxicology Program, while others are still being developed. Nevertheless, some of the published results are encouraging, especially in comparison to the early days of short-term mutagen test development.

Braun and his colleagues (146) have developed an assay that discriminates between teratogens and non-teratogens on the basis of their ability to inhibit attachment of ascites cells to concanavalin A–coated surfaces. This test shows qualitative agreement between animal data and in vitro activity for eighty-one of the 102 (79%) chemicals tested. Mummery and co-workers report that thirty-five of thirty-nine teratogenic and four of eighteen non-teratogenic chemicals interfered with the growth and differentiation of cultured neuroblastoma cells. Eighty-six percent of the chemicals were correctly identified (143).

As is apparent, a number of possible teratogen screening systems can be used for the rapid identification of potential teratogens. At present, no single in vitro system is likely to supersede animal testing, but one can foresee the development of a successful battery of tests for identifying compounds for further in vivo testing. Logistically, it will never be possible to test chemicals in widespread use if we have to rely solely on the mammalian bioassay, which uses small treatment populations and takes six to ten months to complete.

CONCLUSION

It has not been possible in this review to consider thoroughly all possible methods of alternative toxicology testing, but the examination of acute toxicity tests, eye irritancy testing, and fetal toxicity testing should provide a reasonable introduction to the meaning and scope of the concept of alternatives in the field. Greater attention to and support for alternatives to animal testing will not only provide ways to alleviate animal stress and societal pressures, but will in addition bring major benefits to the science of toxicology.

An empirical search for in vitro tests that correlate with various toxic endpoints will not only be insufficient, it will be detrimental. Developing superior methods for safety evaluation will be much more possible if in vitro tests are investigated mechanistically. Cell cultures, both animal and human, will be used to their full potential only when culture techniques are considerably improved. Fully defined growth media that will support the growth of a wide range of defined cells must be developed. It is now possible to maintain and grow many different types of cells that express differentiated function in vitro. For example, changing culture conditions allowed one group of investigators to establish a thyroid cell line that expressed differentiated thyroid cell characteristics even after three years of continuous culture (148). Beating heart cells can be maintained for a week in good condition and have been used to investigate anesthetic (149) and isoproterenol (150) 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 (61, 151, 152).

Current techniques are slowly being improved, refined, and applied where appropriate. Yet even as new tests or approaches become available, a lack of general acceptance can retard their use. In toxicology testing, established methods tend to be set in stone. Even where new approaches seem an improvement and have widespread scientific support, explicit or implicit guidelines are slow to change. Replacing the classic LD50 test with an acute test using fewer animals was proposed in 1968 (44) and has been reproposed many times since, for example.

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 that no longer do what they are meant to and developing new ones that provide better assessments of potential human hazards.

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