

Tiered Testing Strategies—Acute Local Toxicity

Katherine A. Stitzel

Abstract

More work has been done to develop alternatives to animal use in the areas of eye and skin irritation than in any area other than carcinogenicity. There has long been a belief both in the scientific community and among the public that the development of nonanimal tests in these areas should be simple and straightforward. After more than 20 yr of research, we can identify materials corrosive to the skin without using animals, but the assessment of irritation using *in vitro* methods alone is still an illusive goal. This review of current recommendations and industry practices that reduce the number of animals needed for these two tests concludes that animal use for skin irritation testing is not necessary today, with currently available and accepted methodology, except for regulatory reasons. Scientifically sound improvements in current eye irritation methods are also available. Advances in the understanding of the mechanisms of eye irritation that have been made in the last 5 yr should lead to improved *in vitro* methods for this endpoint. In the meantime, changes should be made in the current animal protocol to reduce pain and distress. This paper provides an overview of the progress that has been made toward discontinuing the use of animals in tests to determine the potential of materials to cause skin or eye damage after a single acute exposure. It also discusses some additional changes that could be made now to reduce animal use further or to reduce pain and distress in the testing that must still be done until such time as we can meet the ultimate goal—validated and accepted nonanimal methods for these endpoints.

Key Words: alternative methods; Draize; eye irritation; rabbit; skin corrosion; skin irritation

Progress at the International Level

There has been recent progress toward international acceptance of improved test methods for both eye and skin effects after acute exposure. The Organisation for Economic Co-operation and Development (OECD¹) is in

Katherine A. Stitzel, D.V.M., is an Associate Director of Human Safety at The Procter & Gamble Company, Cincinnati, Ohio.

¹Abbreviations used in this presentation: ECVAM, European Centre for the Validation of Alternative Methods; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; NEISS, National Electronic Injury Surveillance System; OECD, Organisation for Economic Co-operation and Development; SAR, structure activity relationship.

the process of accepting revised test guidelines that include reference to a tiered testing strategy for both of these endpoints (OECD 2001). Revised Guideline 404 will become the method to determine the potential of materials to cause skin corrosion or skin irritation. Revised Guideline 405 will become the method to determine the potential of materials to cause eye corrosion or irritation. These guidelines were accepted by the National Coordinators meeting in May 2001, and final approval is expected before the end of 2001.

Both new guidelines support avoidance of animal testing by taking full advantage of a weight-of-evidence approach to hazard assessment. They reference multistep, tiered-testing strategies developed to help toxicologists use all available information and nonanimal methods to avoid animal tests. When animal testing is absolutely necessary, both revised guidelines recommend performing tests sequentially, one animal at a time, to expose as few animals as possible to corrosive or highly irritating materials. The guidelines also allow immediate euthanasia of an animal if corrosive injury occurs after exposure as well as classification of the test material as corrosive without exposure of additional animals.

Tiered Testing Strategies

The tiered approaches referenced as supplements to the two guidelines are similar and represent a major step forward by applying sound scientific principles to toxicological questions. The initial step in both supplements is a review of all data available on the results of human or animal exposure to the test material. Both guidelines support the use of a weight-of-evidence approach to support classification of the test compound as corrosive, irritant, or nonirritant based on historical data. International acceptance of the use of weight-of-evidence rather than check-the-box approaches to toxicological assessment will help to decrease animal use, and the OECD is to be commended for including this approach in their new guidelines.

Additional steps would be necessary only if data are insufficient to support classification. Although not specifically detailed in the guidelines, a thorough search for all data available on a compound to include as part of the weight of evidence would include information both from previous animal studies and from experience with human exposures, whether occupational or accidental. Sources of data that should be searched include company, supplier and trade association data, and publicly available databases in-

cluding, but not limited to, the Toxic Substance Control Act Test Submissions database of the United States Environmental Protection Agency (<http://esc.syrres.com/efdb/TSCATS.htm>) and the International Uniform Chemical Information Database of the European Chemicals Bureau, which can be purchased for a nominal fee from TechniData AG, Markdorf, Germany. A search of the literature should also be conducted, although the published literature may not contain information on these endpoints.

The revised OECD guidelines also suggest that data on skin irritation or corrosion can be obtained from other animal studies. For instance, it may be possible to determine the irritation potential, or lack of irritation potential, by reviewing the results of a dermal toxicity study. In addition, if a material is highly toxic by the dermal route, it is not necessary to assess its irritation potential also. Although not specifically mentioned, it may also be possible to gain information on the irritation potential of a material from results of studies to assess dermal sensitization or the studies commonly performed to confirm skin safety in humans (Robinson et al. 2002).

For toxicologists to take maximum advantage of every animal study, they need to consider the possibility of collecting data on dermal irritation when designing acute dermal exposure studies including appropriate recording of observations on skin effects. This consideration should include very thoughtful program design, with particular attention to the order in which the toxicity studies that may be necessary are performed. For instance, because according to the OECD guidelines, a positive effect in a skin corrosion study can be used in hazard classification for the eye, skin corrosion studies should be performed before eye irritation studies.

The second step in the tiered schemes—the use of structure activity relationships (SARs¹)—is careful consideration of what is known about other substances with known toxicities that have related chemical structures or similar chemical properties. If the compound is a member of a recognized chemical class and adequate data on other chemicals in that class provide good evidence that all chemicals in the class have similar toxicological profiles, the OECD allows classification of the test material as corrosive or irritating without further animal testing. Several computer programs are available that predict toxicity based on the relationship of a chemical's structure to that of other compounds with known toxicity (e.g., MultiCASE [MULTICASE, Inc., Beechwood, Ohio] and TOPKAT® [Accelrys, Burlington, Massachusetts]). However, none of these programs cover all types of chemicals. Before a decision is made based on a computer program, the toxicologist should be certain that the program has reliable information on structures similar to the test material. The revised OECD guidelines do not allow classification of a test material as negative for these endpoints based on data from SAR programs.

The third step in both tiered approaches takes into account the pH and buffering capacity of the test material. If

the material is highly acidic (pH <2) or highly alkaline (pH >11.5) and has significant buffering capacity, the material can be classified as corrosive to skin or eye without animal testing. Again, negative results cannot be used to classify a material, and the toxicologist moves to the next step in the testing strategy.

In both strategies, the next step involves consideration of whether the compound is highly toxic by the dermal route. If the test material has been found to be highly toxic, then it is not necessary to test the material for skin corrosion or irritation. In addition, if the material was not irritating or corrosive in an acute dermal toxicity test, the material can be classified as nonirritating for skin. This step will be successful in reducing animal testing only if skin effects are adequately observed and recorded in acute dermal toxicity studies and these studies are done before skin irritation tests.

The fifth step in both strategies is to conduct *in vitro* testing for corrosion, assuming a validated and accepted *in vitro* method is available for determining the corrosive potential of the test material. If the material is not shown to be a corrosive based on *in vitro* results, the tiered approach then moves to a validated and accepted *in vitro* method to determine the irritation potential of the test material. Unfortunately, the revised OECD approaches do not allow classification of a material as negative even if a validated and accepted *in vitro* method for irritation exists.

If validated and accepted *in vitro* methods do not exist or if the material would be classified as negative using an *in vitro* assay, the sequential testing strategies then call for animal testing. In both strategies, the first tests would be for skin corrosion because the OECD document recommends that a toxicologist consider a test material corrosive to the eye if it is corrosive to the skin.

If there are no data to estimate the toxicity of the test material or data are available to indicate the material may be corrosive, Test Guideline 404 for skin irritation and corrosion suggests an initial exposure of only 3 min per animal. One should expose and evaluate the area and if the material is judged to be corrosive, no further testing is warranted. If the material is not corrosive, a second patch may be applied to the same animal for an exposure period of 1 hr. If the material is not corrosive after 3 min or 1 hr of exposure, a third patch is applied to the same animal and left in place for 4 hr. If at any time a corrosive effect is observed, the test is immediately terminated and the material is classified as corrosive. If a corrosive effect is not observed, the final patch is removed and the animal is observed for 14 days or until the lesion completely heals, whichever comes first.

If there are data to indicate the material will not be corrosive, only a single patch left in place for 4 hr need be applied to the first animal. In any case, if the material is not corrosive, the irritant or negative response should be confirmed using up to two additional animals. If the material does cause significant irritation, the test guideline suggests exposing these two animals sequentially. If the second animal exhibits the same effect as the first, no additional animals are needed. If at any time the animals show signs of

severe pain or distress, the guideline states the experiment should be terminated. This change makes it unnecessary to keep an animal with a severely painful lesion for 14 days to observe whether the lesion will heal.

Test Guideline 405 for eye irritation/corrosion also suggests conducting testing in a sequential manner if there are no data on the test material or if data indicate the material may be corrosive or highly irritating. The test material is placed in one eye of the first test animal. If the result indicates the material is corrosive or highly irritating, no further testing is necessary. Although animals are to be observed for up to 21 days after exposure for signs of healing, the guideline directs that animals showing signs of severe distress or pain at any stage should be humanely killed. For materials that cause a response equal to or less than a response that would be classified as severely irritating, the guideline directs that the response should be confirmed with up to two more animals, sequentially if the response indicates a strong (or irreversible) irritant effect. If the second animal also reveals a corrosive or severe irritant effect, the test should not be continued. Test Guideline 405, but not 404, also allows toxicologists to euthanize animals humanely 3 days after their lesions have healed, or 3 days after exposure if no test substance effects are noted.

Futher Improvements That Could Be Implemented Immediately

The guidance contained in revised OECD Guidelines 404 and 405 is a significant improvement over the previous guidelines, although further improvements are clearly possible. The first major change is the allowance for toxicologists' use of historical data to make a weight-of-evidence argument to classify the material without further testing. This is the first time an OECD guideline has specifically stated that a weight-of-evidence approach is acceptable in lieu of a specific toxicity test. The OECD should be commended for this major step forward in efforts to reduce animal use by taking advantage of all available data. This change recognizes that it is sometimes possible to develop very strong data on the effects of a new material on humans without performing the specific animal test described in the test guideline. A weight-of-evidence approach also allows for consideration of data developed using new biotechnology tools and the increased understanding of mechanisms of toxicity that will be the inevitable outcome of this work.

To take maximum advantage of this change, we must quickly find ways to increase the availability of both historical toxicology data and nontraditional studies. Because much of the acute toxicity data is not published, we need to find a way to make widely available the information in company files. The current efforts to gather safety data on high production volume chemicals (EPA 2000) and future efforts to gather data on all existing chemicals in commerce (CEC 2001) should significantly increase the amount and the quality of data available to the public on a wide variety of chemical compounds.

Large amounts of data are also available on the results of human exposure to materials. In the United States, for instance, the Consumer Products Safety Commission maintains the National Electronic Injury Surveillance System (NEISS¹) database, which contains results of a stratified sample of emergency room submissions for accidental injuries including chemical exposures. The US Department of Labor Bureau of Labor Statistics also maintains a large database on occupational illnesses, injuries, and fatalities including those resulting from chemical exposure (<http://www.bls.gov/iif/home.htm>). The American Association of Poison Control Centers (AAPCC) maintains a database of exposures as well (<http://www.aapcc.org/poison1.htm>). It is currently difficult, if not impossible, to search for data on specific chemicals; however, efforts should be made to remedy this situation. In addition, the charges for searching the NEISS database for toxicology data before performing animal testing should be reduced or eliminated.

Increasing the amount of available data is not enough; they should be organized so that it is possible to find toxicological data on a new material and/or closely related compounds. Searching by chemical structure is required because structure is the only unique identifier of a compound; names and Chemical Abstracts Service Registry Numbers (CAS numbers) are not specific enough. The International Life Sciences Institute is currently sponsoring an effort to build a database on toxicological endpoints linked to chemical structures without compromising the proprietary nature of the data. The project includes developing the capability of searching for information on chemicals with related structures. Although these two acute endpoints are not included in the current proof-of-concept test, they could be added if this effort is successful. As scientists we also need to encourage publication databases such as Medline to include chemical structure as a searchable field.

A second improvement in the new OECD guideline is the specific mention of the use of data from closely related chemicals. Unfortunately, these guidelines allow the use of these data only to classify materials as corrosive or irritating, not as nonirritating. This stipulation is in contrast to recent US Environmental Protection Agency, European Union Chemicals Bureau, and OECD agreements to allow assessment of the hazards of the high production volume chemicals, including categorization as nonirritating, by using data from other chemicals within the same class (EPA 1999). These changes in regulatory practice, which industry and animal welfare groups welcome, should also be reflected in the OECD Guidelines 404 and 405. Many industries have been using similar methods for many years (Robinson et al. 2002); however, these are new practices for some regulatory agencies, and it will be necessary to familiarize both the regulatory organizations and other industry participants on how judgments can be made successfully using data from related chemicals.

Computer programs developed to predict the toxicity of new materials based on a quantitative SAR are available for these two endpoints (MultiCASETM, MULTICASE, Inc.,

Beechwood, Ohio) (Barratt 1995; Kulkarni et al. 2001). However, it will be necessary to improve and eventually validate them before they can be used to classify materials for regulatory purposes for these endpoints. In the meantime, SAR data should be included in the weight-of-evidence arguments for classification when it can be shown the programs do contain sufficient information on materials with structures similar to the test materials to allow accurate prediction. In the future, other types of computer modeling programs may also be developed. Toxicologists will need to be aware of these programs and apply them to these endpoints as they become available.

The revised OECD 404 Guideline also allows for the determination of skin corrosion by the use of validated and accepted *in vitro* assays. Two systems that use two-dimensional *in vitro* skin culture systems, Episkin™ and Epiderm™, and the transcutaneous electrical resistance assay have been validated by the European Centre for the Validation of Alternative Methods (ECVAM¹) (ECVAM 1999, 2000; Fentem, et al. 1998). All three assays have been accepted by the European Union (ECVAM 1998; EC 2000), and all three assays are under review by the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM¹). Another *in vitro* skin corrosion assay, Corrositex™, has been accepted as valid for specific classes of compounds by ICCVAM (National Toxicology Program 1999) and accepted for use by the US Department of Transportation (DOT 2000). This system has also been accepted as valid for specific chemical classes by ECVAM (ECVAM 2001). The protocol for the transcutaneous electrical resistance assay and a second protocol for the use of two-dimensional skin culture systems are now under consideration by the OECD (OECD 1999). Concerns that had been raised about these three tests were recently resolved at two subsequent OECD Expert Meetings in Berlin, Germany. The agreed-upon revised proposals for the three guidelines will be released shortly for comments. Other national regulatory bodies should rapidly accept these valid tests, which could eliminate the use of animals for skin corrosivity testing.

Currently validated assays do not exist for skin irritation, eye irritation, or eye corrosion. Unfortunately, even when such assays are developed, the revised guidelines do not allow a toxicologist to classify a test material as negative even if such determination is made using a validated and accepted *in vitro* method that can identify negative materials. This position is clearly indefensible. If a validation study has proven that an *in vitro* method can identify non-irritating materials, there is no reason to insist on testing in animals.

The two test guidelines do incorporate the principles of the OECD Humane Endpoints document (OECD 2000). If at any time a material does cause a corrosive lesion, the guidelines allow the animal to be killed immediately and the material is classified as a corrosive without further testing. The guidelines do not yet allow for killing animals exposed to highly irritating materials before the end of the study, but

they both allow euthanasia of any animals showing continuing signs of severe pain or distress. The ability to classify materials even if the animals have been killed before the end of the study is important. It allows the toxicologist to sacrifice animals showing signs of pain and distress without fear of invalidating the study results. Test Guideline 405 allows sacrifice of animals that show no effects of the test materials after 3 days. The 3-day period is necessary to avoid any delayed response. Test Guideline 404 has no provision for early sacrifice of animals that show no effects.

The test guidelines could go farther. First, Test Guideline 405 is very unclear about when it is necessary to test more than one animal if the material is severely irritating. In paragraph 19, it is stated that it is not necessary to test a second animal if the test material causes severe irritation. In paragraph 21, testing up to two more animals is advised if a corrosive response is not seen, and a sequential test is advised for cases in which the first animal has a severe response.

Test Guideline 404 recommends observing the animals for up to 14 days after treatment for signs of recovery. Test Guideline 405 recommends observing the animals for up to 21 days after treatment. Extensive research has revealed that the degree of initial injury of the eye is very predictive of the long-term outcome of the injury (Maurer and Parker 2000; Maurer et al. 1999, 2001a,b). Therefore, the observation period for Test Guideline 405 should be shortened to no more than 3 to 5 days inasmuch as the extent of subsequent recovery is predictable based on the degree of injury at that time. To classify the degree of injury more accurately, eyes with lesions could be examined for pathology at the end of the observation period. Although the author is not aware of similar studies undertaken specifically to answer this question for skin irritation testing, the pathological principles are the same, and it should be possible to assemble scientific justification to shorten the observation period here as well. To shorten the observation period for eye irritation testing, it would be necessary to modify current regulatory classification and labeling schemes that specifically require observation to 21 days and utilize the scientifically based endpoints (area and depth of injury) instead of the less exact number of days-to-clear endpoint currently used.

Neither guideline mentions the use of systemic analgesics or antibiotics during the observation period. The use of systemic analgesics is common in both human and veterinary medicine, and it is time to insist on their use in toxicology tests unless the investigator has a specific scientifically justified reason to withhold this treatment. The same is true of the use of antibiotics when the epithelium has been damaged. Treatment with antibiotics is standard practice for both humans and animals. Toxicology tests are meant to measure the irritation potential of new materials. Allowing lesions to become infected unnecessarily complicates the evaluation of the results in addition to causing unnecessary pain and distress.

There are two major changes in testing strategy not mentioned in these OECD test guidelines that should also be

considered for immediate implementation. The use of rabbits to predict skin irritation has long been known to be problematic because rabbits are not good predictors of the human response (Campbell and Bruce 1981; Nixon et al. 1975; Phillips et al. 1972). Basketter and colleagues (1997) have proposed an acceptable test for the skin irritation potential of new materials using humans. This test uses very small amounts of test materials, previously shown to be noncorrosive, on normal human volunteers for increasing periods of exposure. If or when the volunteer develops a very mild reaction to the test material, the test is stopped. The classification of the material depends on the length of exposure necessary to elicit the mild reaction and is compared with a known positive control material. The positive reaction is very mild—no greater than the irritation response elicited by a variety of practices in everyday life. The developers of the test propose its use only if the test material has been shown not to have systemic effects, if medical supervision exists, and if all of the protections are afforded in a standard regulated clinical trial that includes full informed consent (Robinson et al. 2001). Cultural morays have caused this test to be rejected by some countries; however, for countries that have strong clinical testing guidelines and that wish to use this test, it should be accepted now. Along with acceptance of the new *in vitro* tests for corrosion, the use of the human irritation test will completely remove any need for animal testing for skin irritation.

More than 20 yr ago, the US National Academy of Sciences convened a committee of experts who recognized that the volume of material instilled in the eye in the standard Draize eye irritation test was too large and recommended use of a lower dose (NAS 1977). Subsequent work has shown that one tenth of the volume recommended by the OECD results in a test that is more accurate and more able to separate mild from moderately irritating materials (Freeberg et al. 1984, 1986; Griffith et al. 1980). This test, known as the low-volume eye test, has been successfully used by many companies for surfactant containing materials for more than 15 yr without problems. The test has been shown to predict a more serious response than what is seen in humans for a variety of materials (Cormier et al. 1995). Although it may be necessary to confirm the use of the low-volume eye test for some chemical classes, this procedure should be adopted immediately for use with surfactant materials.

Further Work Is Needed

More needs to be done in the future. Scientists are still working to develop, evaluate, and validate nonanimal methods for skin irritation and eye irritation and corrosion. Until these methods can be developed, we will still depend on the use of animal methods for some new materials when no other information is available. The recent careful characterization of the pathological changes occurring after eye ex-

posure with a variety of materials (Jester et al. 1998; Maurer and Parker 2000; Maurer et al. 1997, 1999, 2001a,b) provides the community with a set of materials of very well-defined irritation potential for future work. This work has also provided a basis for development of future assays that should be able to predict the outcome of an ocular exposure to a test material accurately. Continued work toward gaining acceptance of a clinical test for skin irritation will also be necessary. Finally, the toxicology community will need to utilize the methods and scientific information that is coming from the new molecular biology fields of genomics, proteomics, and metabomics.

Conclusion

Recent revisions in OECD Test Guidelines 404 and 405 offer significant improvements in methods for evaluating skin and eye irritation. More can and should be done, including increased sharing of historical data, increased use of data from accidental human exposures, use of systemic analgesics and antibiotics for animals injured during these tests, shortening of the observation period for both tests, immediate acceptance of the validated *in vitro* methods for skin corrosion, acceptance of the clinical testing protocol for skin irritation, and reduction of the volume of test material in the eye irritation test.

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