

Animal Use in the Safety Evaluation of Chemicals: Harmonization and Emerging Needs

Horst Spielmann

Abstract

An overview is provided of the legal framework in Europe for the use of experimental animals set by European Union (EU) Directive 86/609/EEC and of the activities of EU member states to implement this directive for regulatory testing in animals. The use of animal data in the safety assessment of chemicals by services of the EU Commission and by EU member states is described. Specific examples are given for the current use of animal tests in Europe for the safety evaluation of industrial chemicals, pesticides and biocides, cosmetics, endocrine disrupters, and existing chemicals. Recent progress in implementing the 3Rs concept of Russell and Burch into regulatory guidelines of the EU are described, with particular reference to acute systemic and local toxicity testing. Progress in implementing the 3Rs concept in the EU is compared with the situation outside Europe, particularly with the incentive initiated by the Organisation for Economic Co-operation and Development, to reduce regulatory testing in animals. The harmonization of toxicity test guidelines initiated by the Organisation for Economic Co-operation and Development in 1982 has been the most successful measure to reduce pain and distress of laboratory animals in regulatory testing. From the animal welfare perspective, the international harmonization of test guidelines and the mutual acceptance of data are, therefore, the way forward for all areas of chemicals testing.

Key Words: animal tests; animal welfare; European Union; harmonization; international guidelines; OECD; regulatory testing; toxicity tests

Introduction

In 1959, William Russell and Rex Burch published the book *Principles of Humane Experimental Technique* (Russell and Burch 1959), in which they suggested the “3Rs concept” (refinement, reduction and replacement) for the humane treatment of experimental animals. The 3Rs concept was not recognized for about 20 yr, until the sci-

entific community and the public became increasingly concerned about the use of animals in testing for regulatory and other purposes. The concept was not only rediscovered, but it also has become the generally accepted scientific concept of institutions serving the development of alternatives to animal experiments (e.g., the European Centre for Validation of Alternative Methods [ECVAM¹] and the German Centre for the Documentation and Evaluation of Alternative Methods [ZEBET¹]) in Europe and the Center for Alternatives to Animal Testing (CAAT) in the United States) and of agencies funding the development and validation of nonanimal testing procedures.

In regulatory toxicology, animal tests are used in hazard assessment to identify the toxicological properties of chemicals to which humans or the environment are exposed when the chemicals are used in a specific product or for a specific purpose. Currently, regulatory safety testing of drugs and other chemical entities must be performed in standardized animal tests, first to protect workers during the production of chemical products, and second to protect the consumer and the environment. The exposure of laboratory animals to hazardous chemicals may lead to considerable pain and distress and even death.

To reduce the pain and distress of laboratory animals for regulatory purposes, the 3Rs concept has been applied successfully. The harmonization of test guidelines at the international level has significantly reduced testing in animals, as outlined below. In contrast, it is much more difficult to replace a given animal test used in regulatory toxicology without reducing the safety of chemicals. There are, however, a few examples, which prove that chances are promising to replace regulatory testing in animals if the mechanistic basis of the specific area of toxicology is well understood. In this presentation, the example of using regulatory requirements of the European Union (EU¹) for the safety testing of chemicals is used to illustrate that to date, the international harmonization of test guidelines was the most successful approach to reduce testing in animals for

Horst Spielmann, M.D., is Director and Professor of the National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments (ZEBET) at the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Berlin, Germany.

¹Abbreviations used in this article: ECVAM, European Centre for Validation of Alternative Methods; EEC, European Economic Community; EU, European Union; ICH, International Conference on Harmonization; LD₅₀, lethal dose of a substance that kills half the animals in a test group; LLNA, local lymph node assay; NRU, Neutral Red Uptake; OECD, Organisation for Economic Co-operation and Development; TER, transepidermal resistance; ZEBET, National Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments, Berlin, Germany.

regulatory purposes and that in a few instances, validated nonanimal tests have been accepted for regulatory purposes by EU member states.

Legal Framework in Europe for the Use of Experimental Animals

According to article 7.2 of EU Directive 86/609/EEC on the use of experimental animals,

“an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available” (EU 1986).

Moreover, in the same Directive, it is proposed in article 23 that

“the Commission and member states should encourage research into the development and validation of alternative techniques which could provide the same level of information as that obtained in experiments using animals, but which involve fewer animals or which entail less painful procedures, and shall take such other steps as they consider appropriate to encourage research in this field. The Commission and member states shall monitor trends in experimental methods” (EU 1986).

To promote the implementation of the EU Directive 86/609/EEC on the use of experimental animals, the European Commission and several member states have established centers for the validation of alternative methods (e.g., ZEBET in 1989 at the Federal Health Institute in Berlin, Germany; and ECVAM in 1992 at the Joint Research Centre JRC in Ispra, Italy). Examples of the duties assigned to validation centers are those of ZEBET, which has the following mission:

- to establish a database and information service on alternatives at the national and international level;
- to develop alternatives according to the 3Rs' principles of Russell and Burch;
- to fund research on alternatives;
- to coordinate validation studies;
- to cooperate with national and international funding agencies and validation centers; and
- to provide a forum for information on alternatives to animal testing.

Because ZEBET is part of the Federal Health Administration in Germany, reduction of regulatory testing in animals is ZEBET's main task. Similar missions characterize ECVAM at the EU level and the Interagency Center for the Coordination of the Validation of Alternative Methods (ICCVAM¹) in the United States.

After the establishment of ZEBET in 1989, the annual number of experimental animals in Germany decreased from 2.7 million in 1989 to 1.6 million in 1999. A closer analysis reveals that the decrease between those years was due predominantly to a reduction in the number of animals used for the development of drugs, which decreased 50% from 1.4 to 0.6 million. This dramatic development was due to a general change of the methodology in drug development from animal models to molecular biology and genetics, including cell and tissue culture models. The new technology allowed high-throughput screening of thousands of new drug candidates. This approach is, of course, more predictive, faster, and cheaper than animal models. However, at the same time, it has proven extremely difficult to reduce animal numbers in regulatory testing because the established endpoints in toxicity tests in laboratory animals are usually organ specific and cover endpoints that are quite similar in humans.

Reducing the Animal Numbers in Regulatory Testing by International Harmonization of Test Guidelines

Since the mid-1960s, toxicity testing has been developed empirically in many laboratories around the world. Table 1 provides a summary of toxicity tests currently required for regulatory purposes. For a specific area of toxicology (e.g., eye and skin irritation or embryotoxicity), the standard animal procedures have differed considerably between countries (e.g., species, regimen of treatment, number of animals per treatment group). Differences have also existed in the way the information derived from the animal studies has been used for regulatory purposes (e.g., classification and labeling).

Table 1 Current animal safety tests that must be conducted for regulatory purposes during toxicity testing of chemicals

-
- Acute systemic toxicity (oral, dermal, inhalation)
 - Eye irritation and corrosion
 - Skin irritation and corrosion
 - Skin sensitization
 - Dermal penetration
 - Subacute toxicity
 - Subchronic toxicity
 - Chronic toxicity
 - Toxicokinetics
 - Neurotoxicity
 - Teratology and embryotoxicity
 - Reproductive toxicology
 - Genotoxicity
 - Carcinogenicity
-

Harmonization of the OECD Guidelines for the Testing of Chemicals

The OECD Test Guidelines (OECD 1982, 2001) cover the following four sections: Section 1, physicochemical properties, comprising 20 methods; section 2, effects on biotic systems, comprising 14 methods; section 3, degradation and accumulation, comprising 14 methods; and section 4, health effects, comprising 28 methods on local and systemic toxicity and 15 methods on genetic toxicology.

Differences in national and international test guidelines are unacceptable from the scientific and animal welfare points of view and, most importantly, for economic reasons, because unnecessary or repeated testing places the burden not only on companies operating at the international level, but also on the consumer, who must pay a higher price for the final products containing the tested chemicals. Consequently, industry has insisted on the harmonization of guidelines for testing of chemicals for economic reasons. An obstacle to the harmonization of test guidelines has been hostility in some countries against test procedures developed abroad. In other words, it is the “NIH not invented here syndrome,” which is sometimes still used to protect a marked position. However, for economic reasons, companies required to provide test data to regulators have used flexibility and common sense to convince national and international regulatory agencies that harmonization of test guidelines is the only way forward in a worldwide economy.

In 1982, the OECD (a trade organization and not a scientific institution) was the first international organization to agree on harmonized guidelines for the testing of chemicals. Most importantly, this agreement included mutual acceptance of data produced in member countries of the OECD (the world’s major industrial nations) in studies conducted according to the guidelines for the testing of chemicals (OECD 1982). Since then, a similar approach has been used for the safety and efficacy testing of drugs by the International Conference on Harmonization (ICH¹), which represents the three major economic regions (Europe, Japan, and the United States). Since 1990, the ICH has accepted harmonized guidelines for efficacy and safety testing of drugs and medicines, including animal tests (D’Arcy and Harron 1995). Again, the harmonization of test guidelines has led to significant reduction of testing in animals in that regulatory agencies around the world are now accepting results of tests conducted according to ICH guidelines.

The most important areas that require safety testing in animals and in which the test guidelines have been harmonized at the international level are summarized in Table 2. This summary reveals that in addition to drugs, industrial chemical and pesticide test guidelines have also been harmonized internationally for hormones and biologicals by the pharmacopoeias and for vaccines by the World Health Organization. To date, the harmonization of international test guidelines for toxicity and safety testing has been the most successful approach for reducing animal testing for regulatory purposes.

Table 2 International harmonization of guidelines for toxicity testing in animals^a

- Industrial chemicals, pesticides, cosmetics etc: Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals (OECD 1982-2001)
- Drugs and medical devices: International Conferences on Harmonisation (ICH) (ICH 1990)
- Safety and efficacy of hormones and biological: Pharmacopoeias (European Pharmacopoeia Commission, US Pharmacopoeia)
- Vaccines and other immunologicals: World Health Organization recommendations, European Pharmacopoeia Commission

^aThis summary includes the different fields of safety testing for which international harmonization was implemented for economical reasons (to reduce the cost of testing) and for ethical reasons (to reduce the pain and distress of laboratory animals).

EU Directive 67/548/EEC on the Classification, Packaging, and Labeling of Dangerous Substances

In the EU, official test methods for chemicals are published in Annex V of Directive 67/548/EEC (EC 1967) on the classification, packaging, and labeling of dangerous substances. Annex V (EC 2000a) contains the following methods for the determination of physicochemical properties, toxicity, and ecotoxicity: Part A on physicochemical properties contains 20 methods; part B on toxic properties describes 41 methods, mostly comprising animal tests; and part C on ecotoxic properties contains 13 methods. Part B methods for the determination of effects on human health cover in vivo endpoints of toxicity that are similar to the OECD guidelines for toxicity testing (e.g., acute or chronic toxicity, skin sensitization, irritancy, corrosivity, carcinogenicity, and neurotoxicity). In 2000, for the first time, two in vitro methods for toxicity testing were added to part B: an in vitro method for phototoxicity test and a skin corrosivity test (EC 2000b,c). To keep up with progress in the life sciences, the Annex V methods are continuously updated. At present, 40 test methods of Annex V are under development or revision.

Annex V methods are used for the determination of hazardous properties of chemicals control in the EU, as described in Figure 1. These methods are used for new and existing chemical substances, preparations, plant protection products, biocides, cosmetics, and so forth. The Annex V methods are continuously harmonized with other relevant international test programs (e.g., OECD). Harmonization is conducted through consultation meetings with the EU National Coordinators and Competent Authorities.

Industrial chemicals must undergo a tiered testing strategy before they are marketed. The amount of testing depends on the volume of marketing in the following manner.

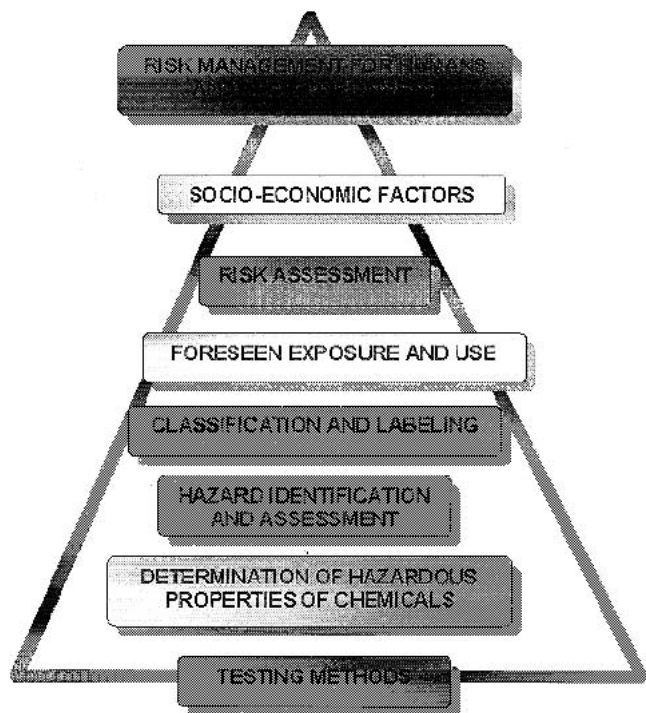


Figure 1 Use of test methods for risk management in Europe. The graph represents test methods published in Annex V of EU Directive 67/548/EEC on the hazard and risk assessment process and also for risk management for humans and the environment.

For stage 1 (production/sales ≤ 100 tons/yr), a base set of testing is required. For stage 2 (production/sales of 100-1000 tons/yr), level 1 of testing is required; and for stage 3 (production/sales > 1000 tons/yr), level 2 of testing is required. This strategy covers the full spectrum of toxicity testing.

The base set (≤ 100 tons/yr) of testing in animals covers the following tests: acute oral or dermal toxicity (depending on exposure), eye irritation/corrosion, skin irritation/corrosion, skin sensitization, subacute toxicity (28 days), and mutagenicity/genotoxicity in nonanimal tests.

Special Areas of Chemical Safety Testing in the EU

Pesticides and Biocides

All animal tests are described in Annex V of Directive 67/548/EEC (see above), including acute and chronic studies in dogs, which are mandatory for pesticides and biocides. For pesticide residues, an acute reference dose study in rodents (lethal dose of a substance that kills half the animals in a test group, or LD_{50}^1) is not yet required but is favored by some regulators, even in EU member states.

From the animal welfare and 3Rs' view points, safety testing in dogs for pesticides and biocides may be reduced

to a single subchronic study. This allowance is the result of an analysis of toxicity data of more than 200 pesticides submitted to the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), the regulatory agency responsible for regulating the safety of pesticides in Germany (Gerbracht and Spielmann 1998; Spielmann and Gerbracht 2001). With regard to the regulation of pesticide residues, there is general agreement at the OECD level that determination of the classical LD_{50} is outdated even for this specific purpose.

Cosmetics (Finished Products and Ingredients)

In 1999, stakeholders in all EU member states unanimously agreed on a voluntary level to stop the safety testing of finished cosmetic products. The involved parties included the European Cosmetic Toiletry and Perfumery Manufacturer's Association (COLIPA), the Scientific Committee on Cosmetology and Non-Food Products (SCCNFP) (the DG SANCO expert committee of the European Commission), ECVAM, and representatives of animal welfare organizations.

Safety testing in animals is still required for new cosmetic ingredients. As mentioned above, the animal tests are described in Annex V of Directive 67/548/EEC (e.g., for local irritation and sensitization); however, a few specific animal tests (e.g., photosensitization/-allergy tests for phototoxicity and photogenotoxicity tests for photomutagenicity) are not covered in Annex V.

Endocrine Disrupters/Hormonally Active Compounds

From a scientific point of view, the in vivo endpoints to be assessed for the regulation of endocrine disrupters both in the human population and in wildlife and the environment have not been defined sufficiently according to the evaluation of the Scientific Committee on Toxicology, Ecotoxicology and the Environment (the expert committee of the DG SANCO of the EU Commission) (CSTEE 1999). Thus, from the European perspective and based on the current scientific evidence, it is not necessary to test for this specific toxic side effect. Moreover, under the current EU regulations for the testing of hormonally active compounds, no specific test has been sufficiently validated to be accepted for regulatory purposes. Finally, from the animal welfare and ethical points of view, EU member states do not accept data produced in nonvalidated, new animal tests for the hazard assessment of hormonally active chemicals/endocrine disrupters regulators.

Existing Chemicals

Since the early 1980s, the EU Commission represented by the European Chemicals Bureau has not been able to obtain sufficient toxicity data for the majority of existing (high-production volume) chemicals from the chemical industry. As a consequence, for many of the existing chemicals, regu-

latory measures such as risk management could not be implemented. This result is unacceptable from the perspective of protecting consumers and the environment.

In 2001, to improve the situation, the EU Commission proposed an identical testing strategy for “new” and “existing” chemicals in the EU White Paper titled *Strategy for a Future Chemicals Policy*. According to the new policy, both new and existing chemicals should be tested preferentially with in vitro methods. This approach will permit testing of the more than 30,000 existing chemicals and new chemicals in the EU within the next 15 yr. Although the new policy has not yet been formally accepted by all EU member states, it appears to be the only way forward from the economical point of view and the expectations of the European citizens.

Taking into account the experience gained from the high-production volume testing of new chemical entities in the drug industry, at a recent ECVAM workshop, regulators and experts from the drug and chemicals industry proposed a tiered testing scheme for existing and new chemicals (Figure 2). According to the new procedure, physicochemical and exposure data must be evaluated before nonanimal and in vitro methods are applied (e.g., as proposed by the ECVAM Integrated Testing Strategies Task Force Report I [Blaauboer et al. 1999]). At specifically defined decision points, the evaluators in industry and regulatory agencies must decide whether a given chemical can be classified and labeled or whether additional testing must be performed in vitro or in vivo (Figure 2). At several stakeholder meetings in EU member states, representatives of the chemical industry have indicated their general agreement to the new chemicals policy although industry will have to carry most of the burden when the new legislation is accepted.

Progress in Implementing the 3Rs Principle into Regulatory Toxicology

EU Test Guidelines for Acute Toxicity Testing

The European Commission (EC 2000a) has accepted two reduction and refinement alternatives into Annex V of EU Directive 67/548/EEC: the fixed dose procedure and the acute toxic class method. In addition, the European Commission and all EU member states have banned the classical LD₅₀ test.

Several validation studies of in vitro alternatives to the Draize eye irritation test have been conducted in Europe since the 1990s. As a result, four in vitro alternatives have been accepted for regulatory purposes to identify materials as severely irritating to the eye according to EU Directive 86/906/EEC for the classification and labeling of hazardous chemicals: (1) the hen’s egg test on the chorionic membrane (HET-CAM) using embryonated chicken eggs on day 9 of incubation; (2) the isolated rabbit eye (IRE) test on eyes of rabbits, which had to be sacrificed for other purposes; (3) the bovine cornea opacity and permeability (BCOP) test on

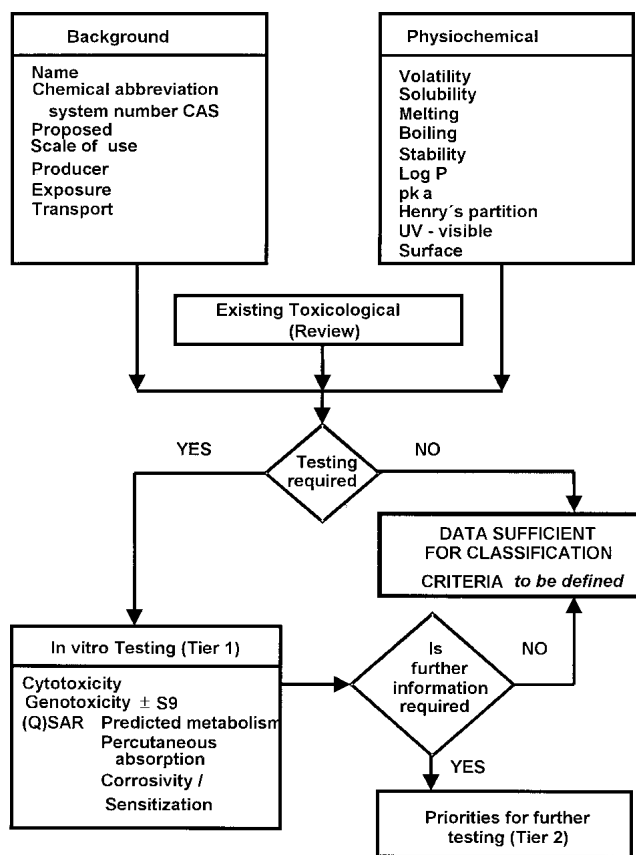


Figure 2 Proposed integrated testing scheme for existing and new chemicals in the European Union (EU). The chart reflects the proposed integrated testing scheme for existing and new chemicals according to the White Paper of the EU Commission for a new chemicals testing policy suggested by participants in a European Centre for Validation of Alternative Methods workshop on “integrated testing of chemicals” held in Angera, Italy, February 12-14, 2001.

the cornea of freshly isolated bovine eyes from the slaughterhouse; and (4) the isolated chicken eye (ICE) test on chicken eyes freshly obtained from the slaughterhouse.

Each of the tests described above has its specific strength and limitations. If a positive result is obtained in one of the four in vitro tests currently accepted by the EU Commission, further testing in animals is not required. Chemicals that provide a negative reaction in any of the four in vitro tests still must be tested in the Draize eye test in one to three rabbits to confirm the absence of eye irritation potential. In several EU member states (e.g., France and Germany), the HET-CAM test is accepted by the national authorities for the safety testing of cosmetics. The described tiered in vitro/in vivo EU testing strategy has reduced the testing of materials that are severely irritating to the eye and has thus reduced the pain and distress of rabbits in the Draize eye irritation test.

The Draize test on rabbit skin to identify severely irritating/corrosive chemicals has recently been replaced by

two in vitro tests in EU member states. In 2000, for the first time, two validated in vitro toxicity test tests have been accepted into Annex V of EU Directive 67/548/EEC, the transepidermal resistance (TER¹) assay, in which conductivity through isolated rabbit skin serves as an endpoint, and the EpiskinTM test, in which a commercial human skin model is used (EC 2000b). When a positive result is obtained in the in vitro tests, further testing in rabbits is not required. A negative result must be confirmed in a Draize eye test with one to three rabbits. The pain and distress of laboratory animals in skin sensitization testing has been considerably reduced since the Magnusen-Kligman test in guinea pigs has been replaced by the local lymph node assay (LLNA¹) test in mice (ECVAM 2000). This test has now been accepted for regulatory purposes in the United States and in Europe and finally (in 2001) by the OECD.

As in the EU in 2001, the OECD has agreed to replace the test for percutaneous absorption/skin penetration in the rat by an in vitro test employing human skin from donors undergoing surgery (OECD 2001). Because the permeability of the human skin for chemicals and finished products may vary across the surface of the body and there is variability among donors, only viable human skin should be used and standardization with control chemicals is essential. The in vitro test for percutaneous absorption is mandatory in international regulations for pesticides/biocides and for cosmetic ingredients and finished products.

Finally, in 2000, the 3T3 Neutral Red Uptake (NRU¹) in vitro phototoxicity test has been accepted into Annex V of EU Directive 67/548/EEC as the first formally validated in vitro toxicity test (EC 2000c). The validation study proved that this in vitro test can detect phototoxic chemicals that are either applied to the skin or used systemically (e.g., as drugs). The 3T3 NRU in vitro phototoxicity test (PT test) is now well established for identifying phototoxic properties not only of chemicals but also of drugs and medicinal products.

OECD Test Guidelines for Acute Toxicity Testing

For acute systemic toxicity, three reduction and refinement alternatives have been accepted into the OECD Guidelines for the testing of chemicals. In addition to the fixed dose procedure and the acute toxic class method, which are accepted in Europe, the up-and-down procedure (UDP) has been accepted into the OECD Test Guidelines (OECD 2001). In addition, by the end of 2000, the OECD had proposed a ban of the classical LD₅₀ test, which had also been banned in all EU member states.

For eye irritation testing, the OECD has suggested (beginning in 1986) that the Draize eye irritation/corrosion test must not be conducted if the chemical is very basic or acidic in solution or if severely irritating/corrosive potential has been identified in a skin irritation test or in a validated in vitro toxicity test. It is quite unfortunate that the OECD has

not yet accepted any of the four in vitro alternatives to the Draize eye irritation test, which are accepted in the EU as outlined above. The tiered testing OECD strategy has significantly reduced the number of rabbits exposed to materials severely irritating to the eye. Negative results must still be confirmed in a Draize eye irritation test in one to three rabbits.

Moreover, in 1998, the OECD accepted a harmonized integrated hazard classification system for human health and environmental effects of chemical substances (OECD 1998). This system includes classification and labeling of eye irritation properties. As a consequence, classification systems in all OECD member states will be harmonized. Results obtained in a Draize eye irritation test and in one OECD member state will also be accepted for regulatory purposes by all of the other OECD member states.

In contrast to the situation in the EU, alternatives to the Draize skin irritation/corrosion test have not yet been accepted by the OECD. However, OECD experts are currently evaluating the proposal of the EU Commission to accept the TER test and the EpiskinTM in vitro test for regulatory purposes. An extended OECD expert consultation meeting is planned to be held by the end of 2001 to decide whether the two in vitro corrosivity tests will be accepted into the OECD Test Guidelines.

The OECD recently accepted the experimentally validated LLNA to assess the skin sensitization potential of chemicals significantly (OECD 2001). This decision is expected to reduce the pain and distress of guinea pigs in skin sensitization testing. The LLNA test is now accepted for regulatory purposes in the United States and Europe and by the OECD.

For percutaneous absorption/skin penetration, the OECD accepted an in vitro test using human skin from donors undergoing surgery (OECD 2001). The in vitro test will replace the in vivo test in the rat, which has specifically been used to assess the percutaneous absorption of pesticides/biocides. The test for percutaneous absorption is mandatory in international regulations for pesticides/biocides and cosmetic ingredients and for finished products.

In contrast to EU member states, the OECD has not yet accepted the 3T3 NRU in vitro phototoxicity test into the OECD test guidelines. An extended OECD expert consultation meeting is planned to be held by the end of 2001 to evaluate the EU Commission's proposal from 1998 to accept the 3T3 NRU in vitro phototoxicity into the OECD test guidelines.

EU/OECD Test Guidelines for Long-term Studies

Regulatory toxicology refinement is the only realistic option in long-term studies. Regulatory agencies and industry must allow the analysis of proprietary data (e.g., the information that can be obtained from dog studies for the risk assessment and management of pesticides [Gerbracht and Spiel-

mann 1998; Spielmann and Gerbracht 2001]) and drugs. Such data may provide sufficient information to reduce studies in dogs for the regulation of drugs and pesticides to a single (subchronic) study.

International harmonization of advanced validated test methods remains essential. The highest hurdle against accepting new methods at the international level has been the “comfort factor” or “NIH not invented here” approach in Europe, the United States, and Japan. In the future, the use of advanced methods, which are generally more humane, should be welcomed and implemented by all international agencies. This approach is the essence of the implementation of the 3Rs’ concept of Russell and Burch into regulatory testing in animals.

Conclusions and Acknowledgment

Since the early 1980s, significant progress has been made in reducing the pain and distress of animals in regulatory testing without reducing the safety of chemicals for the consumer and the environment. Today, we are quite happy that due to the progress in implementing the 3Rs concept of Russell and Burch into regulatory testing in 2000, the OECD has proposed to ban the LD₅₀ test. Finally, it is appreciated that scientists in national and international regulatory agencies and industry, who contributed to this success, have continuously been encouraged and supported by individuals engaged in animal welfare.

References

Blaauboer BJ, Barratt MD, Houston JB. 1999. The integrated use of alternative methods in toxicological risk evaluation. ECVAM Integrated Testing Strategies Task Force Report 1. ATLA 27:229-237.

- CSTEE [Scientific Committee on Toxicology, Ecotoxicology and the Environment]. 1999. Opinion on human and wildlife effects of endocrine disrupting chemicals, with emphasis on wildlife and ecology test methods. <www.europa.eu.int/comm/dg24/health/sc/sct/outcine_en.html>.
- D’Arcy PF, Harron DWG, eds. 1995. Proceedings of the Third International Conference on Harmonization (ICH). Yokohama 1995. Belfast UK: The Queens University of Belfast.
- ECVAM [European Centre for Validation of Alternative Methods]. 2000. Statement on the validity of the local lymph node assay for skin sensitisation testing. ATLA 28:365-367.
- EC [European Commission]. 1967. EU Directive 67/548/EEC on the Classification, Packaging and Labeling of Dangerous Substances. Brussels: EU DG Environment.
- EC [European Commission]. 1986. EU Directive 86/609/EEC 86/609/EWG on the Use of Experimental Animals. Brussels: EU DG Environment.
- EC [European Commission]. 2000a. Annex V of the EU Directive 86/906/EEC for Classification and Labelling of Hazardous Chemicals. Brussels: EU DG Environment.
- EC [European Commission]. 2000b. EU Directive 2000/33/EU for the 21st Amendment of Annex V of the EU Directive 86/906/EEC for Classification and Labelling of Hazardous Chemicals: Test Guideline B-40 “Skin Corrosivity—in Vitro Method.” O. J. European Communities, June 8 2000, L136, p 98-107.
- EC [European Commission]. 2000c. EU Directive 2000/33/EU for the 21st Amendment of Annex V of the EU Directive 86/906/EEC for the Classification and Labelling of Hazardous Chemicals: Test Guideline B-41 “Phototoxicity—in Vitro 3T3 NRU Phototoxicity Test.” O. J. European Communities, June 8 2000, L136, p 98-107.
- Gerbracht U, Spielmann H. 1998. The use of dogs as second species in regulatory testing of pesticides. Part I: Interspecies comparison. Arch Toxicol 72: 319-329.
- OECD [Organisation for Economic Co-operation and Development]. 1982 continuously updated until 2001. OECD Guidelines for Testing of Chemicals. Paris: OECD Publication Office.
- Russell WMS, Burch R. 1959. The Principles of Humane Experimental Technique. London: Methuen & Co. Ltd. [Reissued: 1992, Universities Federation for Animal Welfare, Herts, England.] <http://altweb.jhsph.edu/publications/humane_exp/het-toc.htm>.
- Spielmann H, Gerbracht U. 2001. The use of dogs as second species in regulatory testing of pesticides. Part II: Subacute, subchronic and chronic studies in the dog. Arch Toxicol 75:1-21.