

Future Improvements: Replacement In Vitro Methods

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Abstract

Revolutions in thinking and practice are essential in regulatory toxicology if genuine protection of human beings and the environment is truly to be improved. New test development is the key: Tests should have greater relevance than the current animal procedures based on (1) a mechanistic understanding of the basis of the test method itself and of the toxic phenomenon of concern, (2) taking advantage of new developments in cell and molecular biology and computer systems of various kinds, and (3) a clear understanding of the value of good prediction models. In the not-too-distant future, current research in genomics, proteomics, and metabolomics should provide opportunities for the development of valuable new tests. An inescapable requirement of tests intended to be used for regulatory purposes is validation (i.e., an independent assessment of relevance and reliability for stated purposes according to internationally agreed-upon criteria). However, there is no standard validation scheme; a case-by-case approach is essential. It is important to take advantage of experience, which reveals that prevalidation makes formal validation studies faster, less expensive, and more likely to succeed, and that the procedures for independent assessment used by the European Centre for the Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) are effective in practice.

Key Words: animal testing alternatives; hazard prediction; prediction model; replacement tests; risk assessment; tissue culture; toxicity testing; validation studies

Introduction

The mission of the European Centre for the Validation of Alternative Methods (ECVAM¹) is to play a leading role at the European level in the independent evaluation of the relevance and reliability of tests for specific

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¹Abbreviations used in this presentation: ECVAM, European Centre for the Validation of Alternative Methods; ESAC, ECVAM Scientific Advisory Committee; EU, European Union; ICCVAM, Interagency Coordinating Committee for the Validation of Alternative Methods; OECD, Organization for Economic Cooperation and Development.

purposes, through research on advanced methods and the development and validation of new tests, so that chemicals and products of various kinds can be manufactured, transported, and used more economically and safely, whilst the current reliance on animal test procedures is progressively reduced. This mission reflects the requirements of Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes (Anonymous 1986).

ECVAM provides technical support for other European Commission services responsible for the development of European Union (EU¹) policies and the management of EU legislation. In so doing, ECVAM takes into account the desire of the European Council, the European Parliament, and many citizens and institutions in the member states, for animal testing to be replaced wherever and whenever possible, but without compromising human safety or the protection of the environment (Anonymous 1991; Balls 1995).

Although it is true that this desire is partly driven by animal welfare considerations, the need for better, scientifically more advanced, mechanistically based methods for providing information relevant to human hazard prediction and risk assessment and for protecting the environment is no less compelling. In addition, the alternative methods will also tend to be less expensive to perform, and to have a higher rate of test item throughput.

Thus, there are various good reasons for wanting alternatives to animal tests, including scientific, economic, logistical, ethical, legal, and political pressures. A most satisfying aspect of working in this area is that both humans and animals can be afforded great benefits.

Replacement Alternatives

The overall replacement alternative strategy involves the integrated use of a number of approaches: maximizing the use of existing information (including data concerning the physicochemical properties of chemicals); mathematical predictions based on structure-activity relations and the modeling of physiological, pharmacological, and toxicological processes; experiments on lower organisms (bacteria, fungi, plants, invertebrate animals) and vertebrates at early stages of development (before they become “protected” animals); studies on in vitro systems of various kinds (including whole perfused organs, tissue slices, and cell and tissue cultures); and human studies (including the ethical use of human volunteers, postmarketing surveil-

lance, and epidemiological investigations). My own work has mainly been in *in vitro* toxicology, and, over the last few years, in contributing to the *validation* process (i.e., in working with others to provide independent evaluations of the relevance and reliability of alternative methods for particular purposes) (Balls and Fentem 1999; Balls et al. 1995).

Within the EU, the validation process as managed by ECVAM occupies a pivotal position between research (including the development of new tests and testing strategies) and the scientific and regulatory acceptance and implementation of those tests. However, the general and specific demands and expectations of society are not easily satisfied because there are many fundamental misunderstandings that can sometimes be used by those with vested interests as a means of exploiting and manipulating public opinion. For example, I have recently heard politicians call for “a non-toxic environment” (i.e., an environment free of toxic chemicals) and senior industrialists state that “we must assure the public that chemicals are safe.” However, neither of these fantasies could be delivered because they are impossible. Many chemicals that occur naturally in the environment are innately hazardous, and many chemicals that are manufactured are unavoidably hazardous because of what they are designed to do for our benefit. As for safety, we can only establish the circumstances under which chemicals are *not* safe, and the goal of providing safety for all is as unachievable as that of providing a world in which everybody is happy, there is no disease, and there are no serious accidents. To pretend otherwise is to misunderstand the nature of risk.

Risk is the product of hazard (an inherent property of chemicals or situations) and exposure (the degree, duration, and frequency with which we come into contact with a particular hazard). Therefore, in particular circumstances, risk assessment (and what follows it (e.g., risk management, risk limitation, risk avoidance) cannot be better than the quality of the hazard prediction or of the exposure assessment will permit.

Toxicity tests are intended to provide the data required for a useful hazard prediction to be made, but the routine and uncritical accumulation of mere data is pointless. The term *data* denotes details, facts, figures, statistics, and so forth, whereas what is really needed is *information*, which requires advice, counsel, knowledge, and experience. Such information must then be intelligently and usefully applied.

Improving the Validation Process

Two especially important procedural advances in the validation process have been introduced during the last few years: (1) a *prevalidation study* between test development and a formal validation study, to ensure that tests are truly ready for the rigorous challenge that a well-designed and independently managed validation study should represent (Curren et al. 1995); and (2) the concept of the *prediction model* (i.e., an algorithm for converting the results obtained

with a test system into a prediction of *in vivo* toxicological hazard [Bruner et al. 1996; Worth and Balls 2001]). These achievements have contributed to accelerating the validation process and to reducing its cost, as well as increasing the likelihood that good methods will successfully pass through it. However, there are still those who are unwilling to recognize that test development and regulatory acceptance are truly the more important rate-limiting factors as we seek to replace the current animal tests with more relevant and more reliable alternatives.

Sadly, there are also those who think that a rigorous validation process should be reserved for nonanimal alternative methods, whereas new animal procedures should be readily adopted, merely because they involve animals. Such thinking is unacceptable, both for ethical and scientific reasons and for contradicting common sense. Another problem is that the prediction model appears to be beyond the comprehension of many regulatory toxicologists, who seem content to collect data without worrying about what they really mean or how they should be meaningfully applied.

From our own experience, it is clear that the *scientific* validation process does not need to be long or costly. It is also possible for a newly developed method to join a study already under way, in what we call “catch-up” validation (Balls 1997). Flexibility in light of specific circumstances is the key, provided the highest scientific standards and the necessary degree of independence are maintained.

Validation Successes

ECVAM has been involved in a number of successful validation studies of different kinds: the EU/COLIPA studies on the 3T3 neutral red uptake test for phototoxic potential (ECVAM 1998a) and its application to ultraviolet filters (ECVAM 1998b); the rat skin transepidermal resistance (ECVAM 1998c) and reconstituted human skin (EpiSkinTM, Skin² TM, EpidermTM) tests (ECVAM 1998d, 2000a) for skin corrosivity; three *in vitro* tests for embryotoxic potential (the whole rat embryo culture, micromass and embryonic stem cell tests [Spielmann et al. 2001]); the granulocyte-macrophage colony-forming unit assay for acute neutropenia; and two *in vitro* serological methods (toxin-binding inhibition test and/or enzyme-linked immunoassay) for the potency testing of tetanus vaccines for human and veterinary use (ECVAM 2001a,b). In addition, a number of prevalidation studies on alternative tests for biologicals have been successfully completed for the potency testing of the following: recombinant follicle stimulating hormones, human rabies vaccines, tetanus antisera and immunoglobulins for human use, and leptospirosis vaccines for veterinary use.

Note that ECVAM's work is not confined to chemicals or to the replacement of tests conducted according to OECD guidelines. The Centre's activities also include the development and validation of alternative test methods for pharmaceuticals and biologicals.

The focus of ECVAM prevalidation/validation studies currently in progress includes alternative tests for the following: skin irritation; pyrogenicity; transport across the blood-brain barrier; nephrotoxicity; acute thrombocytopenia; and the specific toxicity testing of diphtheria vaccines with the Vero cell test. Our experience has also convinced us that the proper planning of a validation study is the key to its success. In particular, the number of tests should be as small as possible, the endpoints used should be truly complementary and not duplicative, the number of test items should be as high as possible (but their proper selection is crucial and is best left to an independent committee), and the number of participating laboratories should never exceed four per test.

Regulatory Acceptance

As a result of discussions with ECVAM's customer Directorates General within the Commission and within the ECVAM Scientific Advisory Committee (the ESAC¹, which includes members nominated by the EU member states and the European federations of chemical, cosmetic, and pharmaceutical industry associations as well as members representative of academic in vitro toxicology and animal welfare), an effective system for the independent evaluation and scientific acceptance of ECVAM-sponsored and other validation studies has been set up. The ESAC is kept informed at all stages of a study and receives the final report of the management team for the study after it has been accepted for publication in the peer-reviewed literature. If the conclusion is that a method can be judged to be scientifically valid (i.e., relevant and reliable for a particular purpose), the ESAC makes a statement to that effect (e.g., ECVAM 1998a-d), which is published and communicated to the appropriate Commission services, as well as to others, including the Competent Authorities in the member states, the OECD Secretariat, and the US Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM¹). In the case of biologicals, ESAC statements are also communicated to the European Pharmacopoeia (European Directorate on the Quality of Medicines).

These various authorities then consider the applicability of the scientifically validated method in relation to their own responsibilities, and they may themselves make statements that are published. For example, statements were made by the European Commission's Enterprise Directorate General (Deboyser 1998, 1999) and the Scientific Committee on Cosmetics and Non-Food Products (SCCNFP 1998a,b) on the scientifically validated in vitro methods for phototoxic potential and for skin corrosivity.

Where chemical substances are concerned, acceptance by the other appropriate Commission services can be followed by the submission of a draft guideline to the Competent Authorities for consideration for inclusion in Annex V of Directive 67/548/EEC, the Dangerous Substances Directive (Anonymous 1967). At the same time, a draft guide-

line is sent to the OECD Secretariat for consideration as part of the OECD Test Guidelines Programme.

As a result of this process, the three in vitro methods for phototoxic potential and skin corrosivity were published on June 6, 2000, as Directive 2000/33/EC in the *Official Journal of the European Communities* on (Anonymous 2000), which indicates that they have been accepted as Annex V guidelines for Directive 67/548/EEC and are now the methods that must be used in compliance with EU legislation. The draft guidelines submitted to the OECD Secretariat have been under consideration since October 1998.

ICCVAM has a different process for evaluating the status of alternative test methods and for making recommendations to the US regulatory agencies (ICCVAM/NICEATM 1999). Discussions in progress are aimed at the mutual and bilateral recognition of the ECVAM and ICCVAM procedures for independent evaluation of the validation status of methods. Meanwhile, the ESAC has endorsed the use of the local lymph node assay for skin sensitization (ECVAM 2000b) and of the CORROSITEX[®] method for evaluating the skin corrosivity of certain types of chemicals (ECVAM 2001c), mainly in the light of ICCVAM recommendations on these methods.

What of the Future?

New approaches to validation are necessary for the following reasons: animal test data cannot represent the gold standard to be met by alternative tests; such data are usually lacking, except for a few examples; prospective predictions must replace retrospective predictions; tests are needed for target organ toxicity and chronic toxicity, not just topical toxicity; and new kinds of products (e.g., biotechnology products) must be tested.

Above all, tests based on a sufficient mechanistic understanding are needed. (The term *mechanism* denotes "an explanation of an observed phenomenon, which explains the processes underlying the phenomenon in terms of events at lower levels of organisation" [Frazier 1994], and a *mechanistic test* is "a test based on a system at an acceptable level of organization, with a relevant endpoint based on a sufficient understanding of the cellular or molecular basis of the event under consideration" [Balls 1998].)

First, we need such mechanistic toxicity tests for a variety of reasons: the scientific basis of testing for potential toxic hazard is currently too weak; the current animal and nonanimal tests are mainly correlative, not mechanistically based; developing new correlative tests based on old correlative tests is not good enough; advantage must be taken of developments in fundamental cell and molecular biology; genomics, proteomics, and metabolomics will offer new ways forward; multifactorial phenomena and situations must be dealt with more adequately than at present; and new products (e.g., biotechnology products) represent new challenges.

Second, subject to a caveat, I believe that we could have

an additional and wide range of validated alternative methods within the next 5 yr, including, in the case of chemicals, tests for eye irritation, skin irritation, skin penetration, skin sensitization, hematotoxicology, hepatotoxicity, nephrotoxicity, neurotoxicity, the blood-brain barrier, acute systemic toxicity, and carcinogenesis. The caveat is that this goal will be achieved only if we deal satisfactorily with a number of factors that are already slowing us down, including a lack of: properly developed new test methods for prevalidation/validation, resources for funding development and validation studies, trained personnel able to design and manage validation studies, laboratories with appropriate experience for taking part in validation studies, and reference standard test items backed by sufficient knowledge of sufficient quality; together with an unwillingness on the part of some toxicologists and regulators to accept scientifically validated alternative test methods and testing strategies, and the failure of governments to actively apply legislation requiring the discontinuation of animal test procedures when scientifically satisfactory alternative methods become available.

In fact, however, the situation is much worse than described above. Indeed, there is a trend toward more and more routine check-list animal testing in relation to “endocrine disruptors,” existing high-production-volume chemicals, biocides, pesticides, and food additives, and genetically modified foods.

Securing the Revolution

At the ECVAM Opening Symposium in 1994, I made a number of remarks about the confused and confusing world ECVAM had just joined, including the following:

“Meanwhile, can the OECD and all other agencies involved in the current practice of regulatory toxicology reassure us—indeed, prove to us—that their standards for the acceptance of new animal test procedures are not dramatically less stringent than those which will be applied to nonanimal tests?”

The fact is that they could not provide proof then, and they cannot do so now.

I also made the following comment:

“We need not be hesitant in demanding that scientifically valid and feasible nonanimal methods should be incorporated into regulatory testing strategies. Directive 86/609/EEC specifically requires that ‘an experiment (on an animal) should not be performed, if another scientifically satisfactory method, not entailing the use of an animal, is reasonably and practicably available.’ The law is on our side.”

The law may be on our side, but laws are only as good as the commitment with which they are enforced.

ECVAM and ICCVAM have agreed on the main prin-

ciples of test development—prevalidation, validation, and independent acceptance—and with many partners in industry, academia, animal welfare, and government, we have shown that these principles work in practical real-life situations. Scientifically satisfactory replacement alternative methods have been made reasonably and practicably available.

We thought that agreement on these principles had also been reached with the OECD, at the 1996 Solna workshop (OECD 1996), and in the OECD guidance-drafting group that met in June 1998 to produce a guidance document (which has not yet appeared). More recently, we have learned that the OECD wants to organize another Solna workshop, ostensibly to reconsider the ECVAM/ICCVAM/Solna principles (Balls and Karcher 1995; NIH 1997; OECD 1996). Is this designed to speed up acceptance by OECD Member Countries of scientifically validated methods evaluated and endorsed by the ESAC and ICCVAM? Or is it, as some of us fear, an attempt to weaken the agreed-upon principles in favor of animal testing, principally to smooth the road to acceptance for new animal tests that have not been properly validated (e.g., tests for endocrine disruptors)?

In light of these considerations and concerns, I must confess that I have some doubts about the value of conferences such as the ICLAS/CCAC International Symposium on Regulatory Testing and Animal Welfare. I must say that merely talking about principles and problems is not enough—only effective action really matters in the end.

In closing, then, I turn to *The Holy Bible* and to Christ Himself for support and for a warning:

“Beware of false prophets, which come to you in sheep’s clothing, but inwardly they are ravening wolves. Ye shall know them by their fruits. Every good tree bringeth forth good fruit. Every tree that bringeth not forth good fruit is hewn down, and cast into the fire.” (Matthew 7:15-19).

Unless some of the established trees truly bring forth good fruit, they should be hewn down and replaced by something more effective!

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References

- Anonymous. 1967. Council Directive 67/548/EEC of 27 June 1967 on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Off J Eur Comm 10 (L196) of 16 August 1967:1-98.
- Anonymous. 1986. Council Directive 86/609/EEC of 24 November 1986

- on the approximation of laws, regulations and administrative provisions of the member states regarding the protection of animals used for experimental and other scientific purposes. *Off J Eur Comm* 29 (L358) of 18 December 1986:1-19.
- Anonymous. 1991. Establishment of a European Centre for the Validation of Alternative Methods (ECVAM). Communication from the Commission to the Council and the European Parliament, 29 October 1991. SEC (91) 1794 final. Brussels: Commission of the European Communities.
- Anonymous. 2000. Commission Directive 2000/33/EC of 25 April 2000 adapting to technical progress for the 27th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to their classification, packaging and labelling of dangerous substances. *Off J Eur Comm* 43 (L136) of 8 June 2000:90-107
- Balls M. 1995. Defining the role of ECVAM in the development, validation and acceptance of alternative tests and testing strategies. *Toxicol in Vitro* 9:863-869.
- Balls M. 1997. Defined structural and performance criteria would facilitate the validation and acceptance of alternative test procedures. *ATLA* 25:483-484.
- Balls M. 1998. Mechanistic approaches and the development of alternative toxicity test methods. *Environ Health Perspec* 106(Suppl 2):453-457.
- Balls M, Blaauboer BJ, Fentem JH, Bruner L, Combes RD, Ekwall B, Fielder RJ, Guillouzo A., Lewis RW, Lovell DP, Reinhardt CA, Repetto G, Sladowski D, Spielmann H, Zucco F. 1995. Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM workshop 5. *ATLA* 23:129-147.
- Balls M, Fentem JH. 1999. The validation and acceptance of alternatives to animal testing. *Toxicol in Vitro* 13:837-846.
- Balls M, Karcher W. 1995. The validation of alternative test methods. *ATLA* 23:884-886.
- Bruner L, Carr G, Chamberlain M, Curren R. 1996. Validation of alternative methods for toxicity testing. *Toxicol in Vitro* 10:470-501.
- Curren RD, Southee, JA., Spielmann, H., Liebsch, M, Fentem JH, Balls, M. 1995. The role of prevalidation in the development, validation and acceptance of alternative methods. *ATLA* 23:211-217.
- Deboysier P. 1998. Scientific validity of the 3T3 NRU PT test: Note to ECVAM. *ATLA* 26:386.
- Deboysier P. 1999. Scientific validity of two methods for assessing skin corrosivity: Note to ECVAM. *ATLA* 27:12.
- ECVAM [European Centre for the Validation of Alternative Methods]. 1998a. Statement on the scientific validity of the 3T3 NRU PT test (an in vitro test for phototoxic potential). *ATLA* 26:7-8.
- ECVAM [European Centre for the Validation of Alternative Methods]. 1998b. Statement on the application of the 3T3 NRU PT test to UV filter chemicals. *ATLA* 26:385-386.
- ECVAM [European Centre for the Validation of Alternative Methods]. 1998c. Statement on the scientific validity of the rat skin transcutaneous electrical resistance (TER) test (an in vitro test for skin corrosivity). *ATLA* 26:275-277.
- ECVAM [European Centre for the Validation of Alternative Methods]. 1998d. Statement on the scientific validity of the EPISKIN™ test (an in vitro test for skin corrosivity). *ATLA* 26:277-280.
- ECVAM [European Centre for the Validation of Alternative Methods]. 2000a. Statement on the application of the Epiderm™ human skin model for skin corrosivity testing. *ATLA* 28:365-366.
- ECVAM [European Centre for the Validation of Alternative Methods]. 2000b. Statement on the validity of the local lymph node assay for skin sensitisation testing. *ATLA* 28:366-367.
- ECVAM [European Centre for the Validation of Alternative Methods]. 2001a. Statement on the application of the ELISA procedure for the batch potency testing of tetanus vaccines for human use. *ATLA* 29:93-94
- ECVAM [European Centre for the Validation of Alternative Methods]. 2001b. Statement on the application of the ToBI test for the batch potency testing of tetanus vaccines for human use. *ATLA* 29:94-96.
- ECVAM [European Centre for the Validation of Alternative Methods]. 2001c. Statement on the application of the CORROSITEX™ assay for skin sensitisation testing. *ATLA* 29:96-97.
- Frazier J. 1994. The role of mechanistic toxicology in test method validation. *Toxicol in Vitro* 8:787-791.
- ICCVAM/NICEATM [Interagency Coordinating Committee on the Validation of Alternative Methods/National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods]. 1999. Evaluation of the validation status of toxicological methods: General guidelines for submission to ICCVAM. Research Triangle Park: ICCVAM/NICEATM
- NIH [National Institutes of Health]. 1997. Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods. Research Triangle Park: NIEHS.
- OECD [Organisation for Economic Co-operation and Development]. 1996. Final Report of the OECD Workshop on Harmonisation of Validation and Acceptance Criteria for Alternative Toxicological Test Methods. Paris: OECD.
- SCCNFP [Scientific Committee on Cosmetics and Non-Food Products]. 1998a. Opinion on in vitro methods to assess phototoxicity in the safety evaluation of cosmetic ingredients or mixtures of ingredients adopted by the plenary session of the SCCNFP of 25 November 1998 (SCCNFP/0069/98 Final). Brussels: DG SANCO/C/3.
- SCCNFP [Scientific Committee on Cosmetics and Non-Food Products]. 1998b. Opinion on in vitro methods to assess skin corrosivity in the safety evaluation of cosmetic ingredients or mixtures of ingredients adopted by the plenary session of the SCCNFP of 25 November 1998 (SCCNFP/0070/98 Final). Brussels: DG SANCO/C/3.
- Spielmann H, Genschow E, Scholz G, Brown NA, Piersma AH, Brady M, Clemann N, Huuskonen H, Paillard F, Bremer S, Becker K. 2001. Preliminary results of the ECVAM validation study on three in vitro embryotoxicity tests. *ATLA* 29:301-303.
- Worth AP, Balls M. 2001. The importance of the prediction model in the development and validation of alternative tests. *ATLA* 29:135-143.