Alternatives to Animal Testing in the Quality Control of Immunobiologicals: Current Status and Future Prospects

The Report and Recommendations of ECVAM Workshop 4

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1European Centre for the Validation of Alternative Methods. 2This document represents the agreed report of the participants as individual scientists.
Preface

This is the report of the fourth of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM’s main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of in vitro tests and their potential uses, and make recommendations about the best ways forward.

The workshop on Alternatives to Animal Testing in the Quality Control of Immunobiologicals: Current Status and Future Prospects was held in Utrecht, The Netherlands, on 16–17 April 1994. The conveners were Coenraad Hendriksen and Bernward Garthoff, and the chairman and vice-chairman were Huib van de Donk and Philip Minor, respectively. The participants were representatives from academia, national organisations, international regulatory bodies, and vaccine manufacturers. The aims of the workshop were to evaluate the usefulness of alternative methods for the quality control of immunobiologicals; to identify obstacles to the use of these methods; and to make recommendations concerning future research needs, and about the validation and implementation of alternative tests, in this particular area. These recommendations are summarised in this report.

Introduction

Immunobiologicals are important therapeutic and preventative agents for human and animal health care. During the past century, in addition to improvements in hygiene, diet and housing, the use of these products has resulted in a sharp decline in morbidity and mortality from various infectious diseases. Although interest in new vaccine production strategies is increasing, the production of most vaccines still relies on conventional techniques, involving the attenuation, inactivation or detoxification of the virulent microorganism or toxin to make it suitable for immunisation. Since various steps in the production of vaccines may affect the quality of the resulting product, it is essential to carry out extensive safety and efficacy testing on each batch to ensure that it is both safe and capable of inducing protective immunity following administration.

Most quality control tests are documented in either a compendium (for example, the European Pharmacopoeia) or in guidelines produced by national control authorities and international regulatory bodies, such as the World Health Organisation (WHO) or the Committee for Proprietary Medical Products. Quality control tests utilise about 10% of the total number of laboratory animals used for biomedical research in the UK and in the Netherlands (2, 3). It is estimated that at least ten million laboratory animals are used annually throughout the world for the production and quality control of vaccines.

In the quality control of vaccines, laboratory animals are used either for safety testing or for potency testing. Safety tests are either non-specific (abnormal toxicity test and safety test) or specific (neurovirulence test, mouse weight gain test, and specific toxicity test). Compared with potency tests, safety tests involve only small numbers of animals.

Tests for assessing the potencies of inactivated vaccines are frequently based on challenge models, in which vaccine-induced protection in the experimental animal is assessed by challenge with the virulent microorganism or toxin; the endpoints employed are based on clinical symptoms or lethality. These tests often involve severe distress and suffering of the animals because of the procedures used.

Small numbers of animals are still required for viral vaccine production. These animals generally serve as organ donors. In addition, some animals are needed for the production of monoclonal antibodies, although there is a shift toward the use of in vitro systems.

The interest in alternatives to replace, reduce and/or refine the use of laboratory animals in vaccine production and quality control has grown in the past decade, because of animal welfare, economic, safety and scientific considerations. The participants at
the workshop focused on various opportunities for animal replacement and reduction, as well as for refining animal procedures. Where possible, recommendations have been made to promote the further implementation of the Three Rs in testing requirements. The areas discussed were: a) safety testing — abnormal toxicity tests, safety test for veterinary vaccines, specific toxicity test for diphtheria toxoid, specific toxicity test for pertussis vaccine, and neurovirulence test for oral polio vaccine; and b) potency testing — tetanus and diphtheria toxoids, clostridial vaccines, pertussis vaccine, erysipelas vaccine, leptospirosis vaccine, rabies vaccine, polio vaccine and Aujeszky vaccine.

Guidelines for the Humane Treatment and Care of Animals Used in the Production and Quality Control of Vaccines

Although the number of opportunities to replace animal tests is increasing, it is believed that we cannot replace all animal tests in the near future, in particular those undertaken for quality control purposes. It is recommended that guidelines should be defined for the humane treatment and care of laboratory animals used in vaccine quality control. These are presented in Appendix A. It is envisaged that these guidelines will act as a model for international regulatory agencies and national control authorities, to be adopted as appropriate. In addition, it is hoped that such bodies will specifically consider the design of animal tests, and the application of the Three Rs, when producing and reviewing monographs, product licences, etc. Within their overall objective of requiring animal tests to be carried out to ensure the production of safe and effective vaccines, they might wish to consider animal welfare issues through discrete administrative procedures or committees. This would provide reassurance of the importance such bodies attach to animal welfare issues.

Abnormal Toxicity Test

The purpose of the abnormal toxicity test is to detect any possible contamination in the final lot of product. Two guinea-pigs and five mice are injected with the product (intraperitoneally or subcutaneously) and are observed for seven days. The animals are weighed prior to injection and at the end of the observation period. The test is repeated if one or more of the following criteria are not met: all of the animals survive the observation period; none of the animals show any weight loss at the end of the observation period; none of the animals show toxic signs. If any of these criteria are still not met, the test is carried out a third time, using twice as many animals. A positive result leads to rejection of the batch of vaccine.

The relevance of the abnormal toxicity test was questioned for the following reasons: a) the importance of the test has been considerably diminished by the introduction of Good Manufacturing Practice (GMP) and chemical control tests; b) the test is of a non-specific nature — results are difficult to extrapolate to the target species because of poor correlation with reactions occurring in humans; and c) the test is not mandatory for most pharmaceutical products.

To acquire further information on the relevance or otherwise of the test, an inquiry should be undertaken, preferably by the European Pharmacopoeia Commission. It is recommended that pharmacopoeiae commissions and the WHO:

1. allow regulatory bodies to accept the omission of the abnormal toxicity test for immunobiologics provided that manufacturers have established consistency and characterise each batch with a sufficient set of test methods;

2. where the abnormal toxicity test cannot be omitted, allow the test to be performed on the final bulk instead of on the final lot (this approach has already been adopted in recent European Pharmacopoeia monographs); and

3. consider testing in a single species instead of using two species, since this would facilitate combining the potency and abnormal toxicity tests.

Toxicity Test for Diphtheria Toxoids

This test is undertaken to demonstrate incomplete inactivation of diphtheria toxin or the reversion to toxicity of diphtheria toxoid. The test involves inoculating a group of guinea-pigs subcutaneously with an excess of
the vaccine product, followed by an observation period of about 5–6 weeks.

According to WHO requirements, a tissue culture method is permissible for the determination of residual toxicity in purified diphtheria toxoid. The European Pharmacopeia allows the tissue culture method to be used for investigating the reversion of toxicity, but not for the actual toxicity test.

It is recommended that:

1. the European Pharmacopeia Commission should initiate a small-scale collaborative study to examine the applicability of a tissue culture method for the detection of residual toxicity in purified diphtheria toxoid; and

2. the European Pharmacopeia Commission should allow and promote the use of the tissue culture method for the toxicity test.

However, it was noted that, for technical reasons, control laboratories are rather reluctant to introduce cell culture tests for quality control purposes.

Toxicity Test for Whole-cell Pertussis Vaccine

Reactogenicity of whole-cell pertussis vaccine is believed to be caused by endotoxin and pertussis toxin, and is assessed by the mouse weight gain (MWG) test. In this test, no less than ten mice are injected intraperitoneally with 0.5ml of the final bulk, and another ten (control) animals are injected with 0.5ml saline. The endpoints used are the percentage of the animals that die, and weight gain after 72 hours and after seven days.

In a collaborative study (van Straaten-van de Kapelle, I., van der Gun, J.W., Marsman, F.R., Hendriksen, C.F.M. & van de Donk, H.J.M. Collaborative study on test systems to assess toxicity of whole-cell pertussis vaccine, submitted for publication), it appeared that the relevance of the test was low because of its rather poor ability to discriminate between reactogenic and non-reactogenic products. The alternative test systems evaluated — the histamine sensitising factor test, the Chinese hamster ovary (CHO) cell test, and the lymphocytosis promoting factor (LPF) test for pertussis toxin, and the Limulus amoebocyte lysate (LAL) test for endotoxin — showed large interlaboratory variation, but were somewhat more precise in discriminating between reactogenic and non-reactogenic products. Recently, a capture enzyme-linked immunoabsorbent assay (ELISA), using specific monoclonals to estimate levels of pertussis toxin, has been described. This ELISA could possibly be used as a pre-screening model.

It is recommended that a study should be undertaken in which the estimation of pertussis toxin levels by ELISA and of endotoxin by the LAL test is undertaken in parallel with either the MWG test or one of the alternative test systems. Depending on the outcome of such a study, the ELISA and LAL tests could potentially be introduced as pre-screening models. Threshold levels for this pre-screen would have to be established but, if the data were negative, no functional test would have to be performed on the final bulk. For positive results, one of the functional tests would subsequently have to be performed. With respect to animal welfare concerns, the CHO test or the LPF test are considered preferable.

Safety Test for Inactivated Vaccines

For veterinary vaccines, a safety test conducted in the target species is usually required. This is a double-dose test (for live vaccines, a ten-dose test), in which the vaccine is occasionally administered repeatedly to detect sensitisation. Varying numbers of animals are required, depending on the species used. Although the characteristics of the test are poorly described, it was generally felt to be a relevant test since the effects of the vaccine are directly related to the target species. However, some participants at the workshop considered the safety test to be irrelevant, and the rationale behind the use of a double-dose was questioned. In addition, such a procedure cannot be combined with the potency test (one-dose) in the target species.

It is recommended that:

1. a one-dose safety test should be used and combined, where possible, with the potency test performed in the target animal. A one-dose potency test will in itself generate safety data. For some types of vaccines, a possible alternative which could be evaluated is a double-dose safety test with a double-dose potency test;

2. where the safety test is carried out in the target species and the potency test is
currently undertaken using an animal model (i.e. in a surrogate species), potency tests should be developed in the target species, wherever possible. Validation/calibration work to establish the immune status of the animals in the test would have to be carried out first; and

3. the relevance of repeated testing to establish sensitisation should be evaluated.

Safety Tests for Inactivated Swine Erysipelas Vaccine

At present, two safety tests (double-dose test in pigs and a safety test in mice) are mentioned in the European Pharmacopoeia requirements. The mouse safety test is not considered to be necessary, since sufficient information is obtained by conducting the safety test in the target species. In addition, there is no reason to believe that a second safety test provides more information. Thus, it is recommended that the mouse safety test be omitted from the European Pharmacopoeia monograph.

Neurovirulence Test for Oral Polio Vaccine

The WHO neurovirulence test for oral polio vaccine involves testing each of three serotypes in the vaccine as follows: a group of monkeys (rhesus or cynomolgus) receive samples of each manufactured vaccine lot by intraspinal injection. Another group of monkeys are similarly inoculated with the homotypic reference lot. They are observed for 17–21 days for signs of paralysis, and are then killed and examined for histopathological changes in the central nervous system. The test for polio virus types 1 and 2 requires at least 12 monkeys, and that for polio virus type 3 requires at least 20 monkeys, for each vaccine and reference lot. Proposed alternative approaches for testing of neurovirulence include application of the polymerase chain reaction and the use of a transgenic mouse possessing the human polio virus receptor gene. However, these methods need further elaboration and validation before they can replace the monkey test. Any implementation of an alternative test to replace the existing test is likely to be more than five years away.

It is recommended that:

1. endpoints in the existing test should be reviewed so that they do not include full recumbent paralysis;
2. ongoing collaborative studies which include molecular methods and those involving transgenic animals should be completed and fully evaluated; these should be followed by a second round of studies;
3. larger batches of vaccine should be manufactured, where possible, since this reduces the total need for testing; and
4. the existing test procedure should be re-evaluated in the light of experience gained in the last ten years, to see whether it is justifiable to reduce the number of animals used in each test.

Potency Tests for Inactivated Vaccines

Challenge-type potency assays which are used in the quality control of a number of vaccines are based on animal models which more or less directly represent the mode of action of the vaccine. These models are designed to measure the capacities of the vaccines to protect against bacterial or viral infection, or against the action of toxins responsible for pathogenicity. This is the case for a number of human vaccines (whole-cell pertussis vaccine, inactivated rabies vaccine, and diphtheria and tetanus toxoids) and for several veterinary vaccines (clostridial, leptospirosis, erysipelas, and Ajuszkys vaccines).

It is recommended that, in specific cases, studies should be undertaken to identify and formally validate objective measures for suitable non-lethal endpoints to replace mortality. This would encourage the wider use of such measures and could also lead to replacement of the present official method. As far as possible, such studies should be performed in the context of the statutory potency assays without using any additional animals.

Alternative assays have been, or are being, developed which gradually move away from the challenge-type model: a first step is to measure serological response in vaccinated animals, and a further step is the in vitro measurement of antigen content. As an example, a future perspective on developments in the potency testing of batches of diphtheria and tetanus toxoids is given in Appendix B.

Complete validation of such alternative models is very difficult to achieve. This would
imply detailed understanding of the mode of action of the vaccine, and a reliable prediction of what could go wrong during the production of vaccine batches. Only this understanding would enable the development of models measuring all relevant parameters such as, for serological assays, the right class of protective antibodies and components of cellular immunity and, for in vitro models, the correct epitopes and their presentation. Furthermore, the empirical study of the correlation of potency estimates obtained with challenge-type and alternative models cannot entirely substitute for complete understanding of the mechanisms involved, since it is impossible to study vaccine samples representative of all possible relevant quality failures. Thus, although efforts toward attaining the goal of ideal validation of these alternative tests must be pursued, it is unlikely that it will be achieved in the near future. To speed up the acceptance of alternative tests, and the concomitant reduction in animal testing, it is recommended that new ideas about potential assays are investigated widely. The following are felt to be important in this respect:

1. the suitability of the standard challenge method itself;

2. the possibility of shifting the use of the standard method to stages in the production process before the final bulk, and to perform simplified tests on final bulks or final lots (for example, purified bulk tetanus and diphtheria toxoids; a proposal submitted by Henrik Aggerbeck and Iver Heron [Statens Seruminsitut, Copenhagen, Denmark] is given in Appendix C);

3. the possibility of introducing a system of periodic monitoring, while using both the standard method and routinely performing the alternative test using fewer animals;

4. the degree of characterisation of the product (for example, rDNA products);

5. the available evidence of production consistency; and

6. the use of the standard method by the manufacturer and of the alternative method by the national control authority (or vice versa).

However, whatever approach, or combination of approaches, is taken, no compromise which could jeopardise the quality of the product is acceptable. Potency Test for Diphtheria and Tetanus Toxoids — Single-dilution Assay

In the WHO manual on laboratory methods for testing for the potency of diphtheria, tetanus, pertussis and combined vaccines, the single-dilution model based on a qualitative parameter was discussed as an alternative to the multiple-dilution test, since it requires fewer animals. At the workshop, data were presented on the application of a similar test system based upon a quantitative response parameter. As a preliminary condition, each laboratory wishing to introduce a single-dilution assay based on quantitative parameters should perform a retrospective analysis of the data for the type of vaccine to be tested. This analysis would yield: evidence of consistency in production and testing; evidence of high significance of regression of dose-response data and justification of assumptions of linearity and parallelism; guidance for the selection of the single-dose system parameters; and prediction of the behaviour of the single-dilution system.

The introduction of the single-dilution assay therefore involves a substantial amount of experience with multiple-dilution assays of any given product. Nevertheless, a satisfactory knowledge of the behaviour of the reference preparation may enable a model involving one dilution of the reference preparation and multiple dilutions of the test vaccine to be used. In addition, it is recommended that a system of quality control involving GMP and monitoring of the reliability of the single-dilution test should be introduced. The parameters to be monitored are: mean antitoxin response within the groups of animals; variance of the immune response within the groups; and periodic checking of the performance of the multiple-dilution potency assay (for example, every six months). Depending on the differences between mean antitoxin response to be detected and the acceptable type I and type II errors, the number of animals which need to be used can be derived from statistical tables.

The single-dilution assay is a highly significant approach in reducing the number of animals required and should be given priority. Therefore, it is recommended that:

1. the concept of the single-dilution assay should be introduced in the monographs of the European Pharmacopoeia by analogy with the WHO requirements; and
2. Lethal endpoints should be excluded, either by using serological methods or by using erythrogenic lesions (diphtheria toxoid) or local paralysis (tetanus toxoid) as endpoints.

**Potency Test for Whole-cell Pertussis Vaccine**

Although the acellular pertussis vaccine is currently being evaluated in clinical trials, it is believed that replacement of the whole-cell pertussis vaccine by the acellular vaccine will still take many years. Therefore, attention should be given to a modification of the current potency test. This so-called Kendrick test is based on an intracerebral challenge procedure, using lethality as an endpoint. Large numbers of animals are needed to overcome inherent test variability. The present test could be refined by allowing the humane killing of animals before death from challenge, and this possibility should be mentioned in the European Pharmacopoeia monograph.

Some promising methods are under development as possible replacements for the challenge procedure. These include a serological test based on the assessment of pertussis antitoxin, and a serological test based on the assessment of antibody titres in *Bordetella pertussis* whole-cell ELISA. A good correlation with the mouse protection test is claimed for both methods.

Although the impact of the introduction of a serological approach on the numbers of animals used, and the degree of distress they experience, would be significant, it is recognised that additional validation studies (both intralaboratory and interlaboratory) are needed. It is recommended that:

1. A study should be undertaken to identify and formally validate objective measures for suitable non-lethal endpoints to replace mortality. This would encourage the wider use of such measures, and could also lead to replacement of the present official method;

2. Aspects responsible for variability of the Kendrick test should be identified in order to improve reproducibility; and

3. Studies concerned with the development and evaluation of serological assays should be supported.

**Potency Test for Rabies Vaccine**

While rabies vaccines are formulated by manufacturers on the basis of *in vitro* tests, their potencies are confirmed by an assay which is based on the ability of the vaccine to protect mice from intracerebral challenge with virulent viruses (the National Institutes of Health [NIH] test). A reference vaccine and a titration of the challenge strain are included in the assay. Although rabies vaccine potency tests show poor reproducibility and large numbers of animals are required for the test, this number could, in principle, be reduced without impairing the results of the test (for example, by using a better statistical evaluation of the results).

Several *in vitro* test systems have been developed, based on assessing the antigenic content (glycoprotein) of the vaccine, such as ELISA, single radial diffusion and antibody binding tests. A number of collaborative studies have been carried out in which these *in vitro* methods, or antibody responses in the animal, have been examined for their correlation with the protection assay. No consensus has emerged on a method which can be applied generally. Another approach might be to use the antibody response induced after immunisation.

It is recommended that:

1. The reproducibility of the NIH test should be improved;

2. New challenge routes should be investigated; this should include study of the challenge strain and the route of inoculation. It is recognised that the main cause for concern is the disease itself, not the route by which the challenge strain is given;

3. Correlation of data from *in vitro* and *in vivo* methods should be investigated: for a specific manufacturer, to determine if they can be correlated for an individual product; for the strain of virus used by different manufacturers; and for distinct products;

4. Animals used in immunogenicity studies for immunological responses should be evaluated for correlation with *in vivo* protection;

5. A single-dilution protection test supplemented by *in vitro* tests should be evaluated as a possible replacement for the existing titration method;
6. endpoints other than the death of an animal, for example, involving preliminary symptoms indicative of clinical rabies, should be used; and

7. all available information on the above topics should be collated.

Potency Test for Clostridial Vaccines for Veterinary Use (Excluding Tetanus)

The European Pharmacopoeia contains six monographs on clostridial vaccines and four monographs on antisera. All potency tests are based on a direct or indirect protection test, which requires challenging laboratory animals with toxins or bacterial cultures, resulting in approximately 50% lethality. Various alternative methods, including cell culture assays and ELISA systems, have been developed and successfully evaluated in individual laboratories. However, no collaborative studies have been performed, partly because no reference preparations are available.

For Clostridium perfringens, Clostridium novyi and Clostridium septicum, the animal models described in the monographs are very complicated. Rabbits are vaccinated and bled after a booster immunisation. The pooled serum samples are mixed with toxin solutions and titrated in vivo using mice. A reference serum of equine origin is used to calculate international units (IU). However, there is no information on the protective antibody titre, in IU, for ruminants. The reference preparations are very old and may have lost their potency. Furthermore, the use of horse serum for reference preparations for measuring vaccine potency hinders the validation of alternative methods employing ELISA. New reference preparations which have been tested for potency in the target species are urgently needed.

The potency tests for Clostridium chauvoei and Clostridium botulinum are also challenge models in laboratory animals, but no reference preparations are used. In particular, the potency test for Clostridium chauvoei is extremely painful for the animals involved. Again, no information is available correlating the protection rate in the laboratory animal with the target species.

In recognising the need to replace the challenge tests in the European Pharmacopoeia with serological methods measuring protective antibodies, it is recommended that the European Pharmacopoeia Commission be asked to organise a workshop to examine the following topics:

1. which methods are appropriate to replace the animal challenge methods given in the European Pharmacopoeia monographs?

2. how should these methods be validated?

3. are new reference preparations needed and how should they be tested?

4. which clostridial vaccines should be given priority? It is recommended that coordinated research efforts should be undertaken to establish suitable serological methods for Clostridium chauvoei;

5. since sheep vaccines are mainly multiclstridial products, is it possible to test the potency of all components in only one animal species using serological tests?

6. is information on the protective antibody levels in the target species available?

It is also recommended that objective measures for suitable non-lethal endpoints to replace mortality are identified and formally validated.

Potency Test for Erysipelas Vaccine

The potency test for swine erysipelas is based upon a multi-dilution lethal challenge procedure in mice. Recent data from several groups have provided new information about the protective antigens of Erysipelothrix rhusiopathiae. An ELISA, using a coating preparation that includes these protective structures, has been developed recently at the Paul Ehrlich Institut, Germany. The data suggest that it is possible to replace the mouse challenge model with a serological test. However, before interlaboratory studies can be initiated, it is necessary to establish new reference preparations (standard vaccine, reference sera) and some guidance concerning the design of the study is needed. Fundamental questions with regard to protection have to be answered. So far, no information is available regarding the relationship between vaccine potency (IU) in mice and protection in the target species. Also, further specifications for validation procedures have to be drafted.

It is recommended that:
1. the European Pharmacopoeia Commission should give guidance on validation procedures; and

2. a study should be undertaken with the objective of replacing the multiple-dilution potency test with a single-dilution test, or of reducing the number of animals per vaccine dilution.

**Potency Test for Leptospirosis Vaccine**

The potency test for leptospirosis vaccine is based on a lethal challenge procedure in hamsters. The test is poorly reproducible. Tests based on serology in dogs and guinea-pigs have been developed. However, these models do not correlate with the protection induced. Currently, *in vitro* test systems, estimating antigenic mass using an ELISA, are being studied by the US Department of Agriculture (USDA).

It is recommended that:

1. the European Pharmacopoeia Commission should consider incorporating into the monograph the use of clinical signs as an endpoint, provided that they have been compared with the lethal endpoint;

2. the USDA should be asked to present data on the progress of the *in vitro* test systems; and

3. the use of the guinea-pig model should be evaluated, and the combination of the current procedure with another potency test, for example, on parvovirus, should be considered.

**Potency Test for Aujeszky Vaccine**

According to the European Pharmacopoeia monograph on inactivated Aujeszky vaccine, potency has to be estimated in a lethal challenge test in mice or pigs. The relationship of the challenge test to efficacy in the field is not known. One of the manufacturers has used a serological test in pigs, but no data are available. Further information might be obtainable from a German survey on possible alternative methods (Klaus Cussler, Paul Ehrlich Institut, Langen, Germany).

The European Pharmacopoeia monograph on inactivated Aujeszky vaccine includes the statement that alternative methods might be used, subject to agreement by the national authority. The effects of this statement on harmonisation and mutual acceptance of data were discussed, and it is recommended that:

1. the survey of the German manufacturers concerning the acceptability of existing *in vivo* tests and the development of alternatives, especially *in-house* tests, is made available, and this is examined for acceptable alternatives;

2. manufacturers are encouraged to publish their own alternative methods; and

3. national control authorities are encouraged to harmonise their approach to methods which are alternatives to those explicitly described in the European Pharmacopoeia.

**Potency Test for Inactivated Polio Vaccine**

Various animal models are available for the potency testing of inactivated polio vaccine. The officially recognised tests include sero-response models in guinea-pigs and chickens. Both methods are difficult to standardise and show large interlaboratory variation. Some laboratories rely on a serological rat model and good results have been reported.

An *in vitro* method currently being investigated is an antigenic mass test: the D-antigen test, based on ELISA. In a collaborative study carried out by the US Food and Drug Administration, which employed a standardised ELISA kit, it appeared that there was poor correlation between the *in vitro* and the *in vivo* data. A second collaborative study, which is being conducted under the auspices of the WHO, is under way. The relationship between results from the D-antigen test and vaccine efficacy in animals and humans should be elucidated.

It is recommended that:

1. results of the collaborative studies on *in vitro* tests should be evaluated;

2. consideration should be given to assaying the immunogenicity in any single suitable animal species where national requirements differ; and

3. the serological responses relevant to protection should be examined.
Validation

The workshop participants felt that guidance should be given with respect to validation procedures, to facilitate the acceptance and introduction of alternative methods, particularly in vitro methods. A flow-chart for validation was discussed at the workshop. Of particular importance is the availability of reference preparations and reference antisera. Also, the production of preparations of poor quality, as a reference for evaluating the reliability of the alternative test, was proposed. Intralaboratory studies should be followed by interlaboratory studies, and a broad range of products should be included.

It was concluded that general rules for validation procedures cannot be given, that protocols should be drafted on a case-by-case basis. It is the responsibility of the individual manufacturer to evaluate the use of the alternative and validated models for his kind of products. It is recommended that, following intralaboratory validation projects on reduction, refinement and replacement alternatives, the results should be submitted to the European Pharmacopoeia Commission. The feasibility of the alternative test should then be evaluated by consultation with a number of experts. If the outcome is positive, the European Pharmacopoeia Commission should subsequently initiate and provide guidance for an interlaboratory validation.

Harmonisation

Harmonisation of guidelines at an intercontinental level was considered to be an essential step toward further animal reduction. Harmonisation should be laid down in a treaty on the mutual acceptance of data, although such a treaty would be considered to be a political, rather than a technical, commitment.

Summary of Recommendations

Recommendations to ECVAM

1. Provide financial support for studies directed toward identifying humane endpoints in lethal protection tests (in order of priority: pertussis vaccine, rabies vaccine, clostridial vaccines, leptospirosis vaccine, erysipelas vaccine, Avian flu vaccine).

2. Establish a task force or workshop on clostridial vaccines.

3. Provide financial support for collaborative studies (for example, on erysipelas and pertussis vaccines).

Recommendations to the European Pharmacopoeia Commission

4. Consider and implement the guidelines for the humane use and care of laboratory animals (Appendix A).

5. Draw up guidelines for interlaboratory validation procedures, and initiate and coordinate these studies. Continue the introduction of international working reference preparations.

6. Review and modify monographs to incorporate the Three Rs (replacement, reduction and refinement) concept.

7. Omit the present animal tests for abnormal toxicity, where possible, or continue replacing the test on the final lot with a test on the final bulk, and re-evaluate the use of a second species.

8. Omit the double-dose safety test, and combine the potency test in the target animal with the safety test.

9. Identify clinical endpoints to replace lethal endpoints in protection tests, and modify the monographs accordingly.

10. Accept single-dilution tests for diphtheria and tetanus toxoid vaccines.

Recommendations to the WHO

11. Consider and implement the guidelines for the humane use and care of laboratory animals (Appendix A).

12. Set guidelines for interlaboratory validation procedures, and initiate and coordinate these studies. Initiate production of reference preparations.

13. Review and modify monographs to incorporate the Three Rs concept.

14. Omit the present animal test for abnormal toxicity, or replace the test on the final lot with a test on the final bulk, and evaluate the use of a second species.

15. Identify clinical endpoints to replace lethal endpoints in protection tests.
Recommendations to vaccine manufacturers and the scientific community

16. Re-evaluate data from in vitro tests and undertake correlation studies with the rabies in vivo potency test.

17. Evaluate the rabies immunogenicity test, and investigate the potential for combining serology measurements with in vitro tests as a routine potency test.

18. Evaluate the specific in vitro toxicity test for diphtheria toxoid vaccine.


20. Implement the guidelines for the humane use and care of laboratory animals (Appendix A).

References

Appendix A: Guidelines for the Humane Treatment and Care of Animals Used in the Production and Quality Control of Vaccines

Although the number of research activities concerned with the replacement of tests which involve the use of animals is increasing, it is believed that we cannot do without animal tests in the near future, in particular for quality control purposes.

National and international laws exist for the protection of laboratory animals. Within Europe, two important statements are the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (1) and Directive 86/609/EEC (2). Article 23 of Directive 86/609/EEC states 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" (2).

General Recommendations

The following recommendations are based, in part, on those given by Covino et al. (3), Howard-Jonas (4) and Acrod et al. (5).

1. Whenever possible, validated procedures which do not use animals at all should be employed (2, Article 7).

2. It is a legal and ethical obligation for all those involved in vaccine production and quality control to have a humane regard for their animal subjects, to prevent as far as possible pain and distress, and to be constantly aware of the possibilities of achieving the same result without resort to living animals (2, Articles 5, 7 and 23).

3. An expert in laboratory animal science/medicine (veterinarian) should be responsible for animal health care. Animal caretakers and animal technicians involved in vaccine production and quality control should have proper schooling and additional training in infectious models. They should be familiar with common signs of infectious diseases, as well as with signs of pain, suffering and distress (2, Articles 14 and 19).

4. It is the responsibility of the management to ensure that employees involved in conducting procedures using animals have appropriate qualifications and/or experience (2, Articles 7, 16 and 19).

5. Only clinically healthy animals should be used (5). Animals being used in an experiment should be observed daily for their general condition and for signs of pain, suffering and/or distress. If there is an increased risk of adverse effects or disease, animals should be observed at least twice a day (2, Article 5).

6. Animals should be properly housed under species-specific conditions. Steps should be taken to improve housing conditions by environmental enrichment appropriate to the species. In particular, animal cages should be of an adequate size and appropriate bedding material (sawdust or tissues) must be used. However, housing conditions should not interfere with the observations to be made during the experimental procedure (2, Articles 5 and 19).

7. Animals should be adequately supplied with food and water. This must be readily accessible and for infected mice, for example, moistened bread could be supplied (2, Articles 5 and 19).

Experimental Design

1. In order to ensure consistency in testing and to avoid unnecessary duplication, procedures using laboratory animals should be performed according to Standard Operating Procedures and nationally implemented laws (2, Articles 7, 19 and 22).

2. The minimum number of animals per group and the minimum number of dose levels should be employed, in line with the level of confidence required. Information on minimising the number of animals required may be obtained by retrospective analysis of data and/or by statistical analysis (2, Articles 7, 19 and 22).
3. Critical events and adequate measures for reducing the associated pain and suffering of the animals should be identified. Clinical symptoms of experimentally induced diseases should be recognised early and, whenever possible, measures to reduce suffering should be taken without affecting the experimental design (2, Article 8).

4. Whenever possible, the lethality endpoint should be replaced with other parameters. In potency testing, preference should be given to validated in vitro serological systems. When challenge of experimental animals is unavoidable, clear clinical symptoms should be regarded as equivalent to the lethality endpoint and animals should then be killed humanely (2, Article 8).

5. Humane endpoints should be kept under constant review and refined in the light of experience.

6. The use of in vitro methods for small-scale production of monoclonal antibodies should be encouraged. Bulk production and the production of monoclonal antibodies for therapeutic purposes by the mouse ascites technique should not be permissible.

References


Appendix B: Potency Tests for Diphtheria and Tetanus Toxoids.
Future Developments Incorporating the Three Rs

Current Situation

Each batch of vaccine should be tested for potency as follows:

a) for diphtheria toxoid — by lethal or intradermal challenge in guinea-pigs;

b) for tetanus toxoid — by lethal or paralytic challenge in guinea-pigs or mice.

Future Developments

Short-term

1. Replacement of lethality by humane endpoints.

2. Introduction of a multi-dilution bioassay based on antibody titration in a serological test system:

   a) for diphtheria toxoid — haemagglutination, ELISA, toxin-binding inhibition (ToBI) or Vero-cell assays;

   b) for tetanus toxoid — haemagglutination, ELISA or ToBI assays.

3. As a result of GMP, consistency in the procedures employed in the production and quality control of vaccines can be demonstrated. A single-dilution assay based on serology should be introduced.

Long-term

Depending on the batch size, several batches of diphtheria and tetanus vaccines could be made from the same bulk toxoids. The following should be undertaken.

1. Characterisation of the bulk toxoid (e.g. using monoclonals).

2. Potency estimation, using a single-dilution assay, of every first batch of vaccine produced from the bulk toxoid.

3. Determination of the Limes flocculation (Lf) content.

4. Measurement of the degree of adsorption by determining the Lf content of the supernatant.
Appendix C: Potency Tests for Diphtheria, Tetanus and Combined Vaccines. Suggestions for Simplified Assays

Background

Two diphtheria and tetanus vaccines adsorbed to either aluminium hydroxide or calcium phosphate, but identical with respect to toxoid origin and amounts, have been compared by in vivo potency assays in animals and in humans. Potency assays were carried out in mice and in guinea-pigs using standard procedures described in the European Pharmacopoeia, by two-fold dilution of doses in saline. They were also carried out by dilution of doses in adjuvant, and by the injection of one or two doses of undiluted vaccine. The results of the various potency assays showed that the adjuvanticity of calcium phosphate was lower than, or equal to, that of aluminium hydroxide. In humans, however, the highest antibody response was found in the group revaccinated with calcium phosphate-adsorbed vaccine (Aggerbeck, H. & Heron, I. Adjuvanticity of aluminium hydroxide and calcium phosphate in diphtheria and tetanus vaccine, submitted for publication). Nyerges et al. (1) and Relyveld et al. (2) have also found no correlation between the results of potency assays in animals and antibody responses in humans.

In recent years, pilot vaccines have been made from all bulk purified toxoids in the control laboratory at Statens Seruminstitut (Copenhagen, Denmark) and have been tested in potency assays. Final vaccines, including diphtheria, tetanus, and combined diphtheria and tetanus vaccines, made on a production scale from toxoids previously tested in this way, have all passed the requirements relating to vaccine potency given in the European Pharmacopoeia. A logical consequence of this would be to stop doing full potency assays on all final vaccine formulations, but instead perform such an assay on a pilot vaccine made from bulk purified toxoid. Lyng & Heron (3) have used a different approach to suggest a comparable design, which would similarly reduce the number of animals required while still being suitable for demonstrating consistency of production and for identifying toxoids of inferior quality.

Assay Design

1. A pilot vaccine is made from the bulk purified toxoid so that it contains the same amounts of antigen and adjuvant as the reference vaccine.

2. Three-dilution potency assays are carried out in mice or guinea-pigs.

3. Scores are based on antibody titres or on survival rates.

4. The toxoid is released for all final vaccine formulations if the lower confidence limit of the relative potency is not less than 30IU/human dose for diphtheria, or 40 IU/human dose for tetanus.

5. The immunogenic power per Lf, as well as the antigenic power, should be closely monitored.

6. Final vaccine formulations are tested in a simplified potency test by injecting about ten guinea-pigs with an undiluted fraction of a human dose. The same serum samples can be used to test the antibody levels against the different antigens using in vitro assays (for example, by ELISA and by the Vero-cell assay). A similar design has already been described and accepted for testing veterinary tetanus vaccines (4).

References


Appendix C was written by Henrik Aggerbeck and Iver Heron (Statens Seruminstitut, Copenhagen, Denmark).