

Humane endpoints in vaccine research and quality control

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Summary

Animal tests are the basic experiments to demonstrate efficacy and potency of vaccines. Large numbers of laboratory animals are needed especially for routine batch testing. For veterinary vaccines it is also necessary to use animals of the target species at least during the development of a new product. In general, legislation controlling animal research requires scientists to select procedures that cause the least suffering for the animals, which clearly indicates that humane endpoints should be used whenever possible. However, many monographs on animal testing of vaccines specify vaccination-challenge models which define survival or death of the animals as the evaluation criterion. An overview of the regulatory requirements for challenge tests and recommendations for the introduction of humane endpoints is given. The potency test for rabies vaccine in laboratory mice is used to demonstrate a possible strategy for improvements in animal welfare.

Vaccines are made from inactivated or attenuated microorganisms and their production is a highly variable biological process. Therefore it is much more difficult to get a consistent production for biologicals than it is for pharmaceuticals. Microorganisms not only consist of antigens inducing protection but may often include or produce harmful substances which could lead to unwanted side effects in the vaccinee. At the beginning of the era of immunization, accidents with ineffective antisera or unsafe vaccines clearly showed the possible risks, and so high standards are now required for quality control, i.e. animal tests have to guarantee safety as well as potency.

Animals have to be used because vaccines are intended to stimulate the immune system, and at the present time this can only be studied in whole animals. Furthermore, for veterinary products, immunogenicity and efficacy studies have to be conducted in the species for which the vaccine is intended (Lensing *et al.* 1995, Council of Europe 1997) whereas for human vaccines, animal models

are used during both the development of a product as well as testing every batch for safety and potency. The responsible national authorities authorize the release of biologicals but on occasions may repeat the testing. Consequently, many laboratory animals are used for routine batch release of vaccines.

Small rodents are highly susceptible to many infections, and challenge with virulent cultures or toxins is often used to demonstrate the protective effect of a vaccine. For some products it is required to calculate the potency in International Units (IU) by means of the classical multiple-dilution assay. Such quantitative vaccination-challenge tests are mainly used for inactivated vaccines such as tetanus, diphtheria, pertussis, rabies and erysipelas vaccines, and at least 100 animals are required to test each batch of these vaccines (Weisser & Hechler 1997). The assays also have to be performed in such a way that more than half the animals succumb to infection to ensure fulfilment of the evaluation criteria. For animal welfare reasons, therefore, such animal tests are of great

concern and refinement alternatives to minimize animal suffering are urgently needed.

The Three Rs concept on alternatives to animal use, developed by Russell and Burch (1959), applies equally to vaccine testing as to other areas of research, but unfortunately the concept has not really been implemented to any great extent. However, during the last decade the situation has changed rapidly in the area of immunobiologicals. Nowadays considerable effort is put into replacement and reduction alternatives, but due to the long period necessary to introduce and validate alternative techniques, progress to change the test requirements has been limited. In the meantime challenge tests will continue to have to be used to estimate the potency and safety of certain types of vaccines but we advocate that much more emphasis should be placed on the refinement of vaccine testing, especially when severe suffering is involved.

Vaccination-challenge tests usually compare the death rate in vaccinates with controls, and a possibility for refinement is through reducing the duration of the animal tests in order to shorten the period of suffering. For example, the humane killing of animals should be permitted when clinical signs of infection are seen. With the exception of tetanus vaccine tests (see below), clinical signs have not been used as endpoints in rodent models and we believe it would be productive to evaluate the use of clinical signs as humane endpoints in order to minimize animal suffering.

Requirements for vaccine potency tests

The *European Pharmacopoeia (Ph.Eur.)* provides the scientific and technical standards for the quality control of medicinal products, including immunobiologicals. In the United States similar requirements are to be found in the Code of Federal Regulations (CFR) where Title 9 refers to veterinary immunobiologicals and Title 21 to human vaccines (USDA 1998). These regulations are legally binding. In addition, other international organizations, such as the World Health Organization

(WHO) and the Office International des Epizooties (OIE), issue guidelines for biological substances, and while not legally binding they are much respected in terms of international trade.

Humane endpoints and vaccine potency tests

Pharmacopoeial requirements are not exempt from complying with animal welfare legislation, for example, the European Convention Treaty No. 123 and Directive 86/609/EEC explicitly refer to the Three Rs concept. Although the *Ph.Eur.* states that:

'... with agreement of the national authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used',

it appears very difficult to obtain permission from every national authority. In addition, each manufacturer would have to validate a variation from the *Ph.Eur.* method separately. There is also the perception that the introduction of humane endpoints would increase the cost of animal testing considerably, which is likely to discourage manufacturers from introducing humane endpoints. This is supported by the results of an informal survey of vaccine manufacturers and national authorities on the use of humane endpoints (C. F. M. Hendriksen, unpublished results). It appears that almost all laboratories use death as the endpoint as laid out in the monographs. Death as an endpoint is attractive to many scientists as it is objective and not time-consuming, whereas the use of humane endpoints requires frequent and careful observation of all animals. Keeping in mind the large animal numbers needed for certain tests, it does not seem very realistic to expect the adoption of humane endpoints on a voluntary basis. It is, therefore, very important to mandate humane endpoints to be used in official

testing requirements, such as pharmacopoeial monographs. For example, the USDA has recently introduced a new paragraph in 9CFR (USDA 1998) requiring the introduction of humane endpoints: § 117.4 (e)

'Test animals that show clinical signs of illness that are due to the test may be treated or humanely destroyed if the illness has progressed to a point ... when death is certain to occur without therapeutic intervention. When interpreting the results of the test, the animals that were treated or humanely destroyed ... and the animals that have died from illness due to the test prior to being humanely destroyed shall be combined into a common statistic of mortality due to the test.'

Other regulatory bodies should follow this example and introduce a similar requirement.

Opportunities to use humane endpoints in potency testing

Depending on the vaccine, many different animal models are used but most of them originate from diagnostic procedures. The kind of infectious agent, the laboratory animal species and the route of infection all influence the course of a disease and so the possibility of using humane endpoints largely depends on the reliable observation of typical clinical signs, as well as other relevant parameters such as variations in body weight and body temperature (see Acred *et al.* 1994). Score sheets of clinical signs and other relevant information should be developed for all models of infection (Morton 1998, 1999 Hendriksen *et al.* 1999).

For some animal challenge tests this will lead to a considerable reduction in animal suffering, but in others it may have little impact. The following examples of different diseases illustrate these points.

Example 1: The tetanus vaccine challenge test

The potency of a tetanus vaccine batch is determined by comparing the dose of vaccine required to protect guinea pigs or mice from

the effects of a subcutaneous injection of a lethal or paralytic dose of tetanus toxin with the dose of a reference preparation needed to give the same protection. The monograph explicitly permits paralysis to be an endpoint. Furthermore, it is stated that the mice should be examined twice daily, and that all animals showing definite signs of tetanus paralysis should be removed and humanely killed. It was striking, therefore, to note in Hendriksen's informal survey that manufacturers and national control authorities, even in the case of the tetanus potency test, preferred lethality as an endpoint. This is not acceptable from the viewpoint of animal welfare. The explicit allowance of lethality as a criterion should be deleted from this monograph. Further refinement should also be made by requiring the use of a different site for injection: for diagnostic purposes the injection is made at the base of the tail which results in a typical paralysis of the tail at an early stage of the disease (Olds 1975).

Example 2: The rabies vaccine challenge test

It is a worldwide requirement that batches of inactivated rabies vaccines for human and veterinary use undergo a mouse challenge test. The recent revision of the *Ph.Eur.* monograph for rabies vaccines (inactivated) now allows alternative methods for the first time but only for veterinary use. We have been investigating the potential for introducing early humane endpoints in routine vaccine control tests performed according to the *Ph.Eur.* monograph (Cussler *et al.* 1998) by observing clinical signs as well as body weight and body temperature. Due to the extra work and the additional cost of temperature transponders, only animal groups with an expected lethality rate of around 50% were used in this experiment. In brief, groups of mice were vaccinated with dilutions of the vaccine or the reference preparation. Additional groups of unvaccinated control animals were used to calculate the challenge dose given intracerebrally. Body temperature, body weight and clinical signs were measured and recorded on score sheets.

The clinical signs of rabies infection in mice are listed and grouped in Table 1 and the results given and analysed in Table 2. The first non-specific signs of the disease such as a ruffled fur appeared around day 5, with typical signs of neurological disorder (scored 2 or higher) following over the next few days. The most important observation was that animals scoring 2 or higher never recovered.

The monitoring of body weight showed an early decrease before clinical signs of the disease were observed (see Table 3). This weight loss continued and could reach 30–40% by the time the animal died. The measurement of body temperature gave surprising results. In contrast with the development of the disease in humans, where an increase in body temperature is one of the early non-specific symptoms of rabies, no significant

increase was ever observed in mice. A decrease in body temperature was evident in the final stages of the disease but only after clinical signs and the decrease in body weight had significantly changed.

In conclusion, the rabies challenge test offers a very good opportunity to introduce humane endpoints. A decrease in body weight of more than 15% in combination with definite clinical signs of neurological disorder would shorten an experiment on average by 3 days (Cussler *et al.* 1998). We recommend, therefore, that the first appearance of typical neurological disorders (showing slow circular movements), scoring 2 or more, become the endpoint for this test.

Example 3: The erysipelas vaccine challenge test

Swine erysipelas is a bacterial disease and vaccines are currently tested for potency in a multiple-dilution challenge assay in mice (Council of Europe 1997, USDA 1998). Animals are vaccinated subcutaneously and 3 weeks later are challenged with an intraperitoneal injection of virulent bacteria sufficient to kill all unprotected control animals. As with the rabies test, body weight, body temperature and clinical signs were evaluated in representative groups of mice.

Table 1 Clinical signs of rabies in the laboratory mouse

Clinical signs	Score
Ruffled fur, hunched back	1
Slow movements, circular movements	2
Trembling, shaky movements, convulsions	3
Lameness, paralysis	4
Prostration, permanent recumbency	5

Table 2 Score of clinical signs of rabies in a group of mice

Day p.inf.	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7	Mouse 8	Mouse 9	Mouse 10
1	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	1	1	1	2	1	0	0
7	2	0	0	1	2	0	2	3	1	0
8	5	0	0	1	4	0	4	4	1	0
9	–	0	0	2	5	0	5	5	1	0
10	–	0	0	4	–	0	–	–	3	0
11	–	0	0	5	–	0	–	–	–	0
12	–	0	0	–	–	0	–	–	–	0
13	–	0	0	–	–	0	–	–	–	0
14	–	0	0	–	–	0	–	–	–	0
15	–	0	0	–	–	0	–	–	–	0

0 = normal; – = animal died

Table 3 Development of clinical parameters in mice after rabies infection

Mouse 4												
Day	1	2	3	4	5	6	7	8	9	10	11	12
Clinical signs	0	0	0	0	0	1	1	1	2	4	5	
Body weight	27.1	26.7	26.1	26.2	25.7	23.5	21.7	20.1	18.3	17.3	15.8	
Temperature	38.5	38.4	39.3	38.6	39.0	38.0	37.6	37.4	37.4	36.6	27.4	
Mouse 7												
Day	1	2	3	4	5	6	7	8	9	10	11	12
Clinical signs	0	0	0	0	0	2	2	4	5			
Body weight	24.6	22.3	22.7	21.8	20.3	18.7	17.1	15.4	13.8			
Temperature	37.6	37.7	37.3	39.0	37.4	37.3	35.2	32.1	25.5			
Mouse 8												
Day	1	2	3	4	5	6	7	8	9	10	11	12
Clinical signs	0	0	0	0	0	1	3	4	5			
Body weight	28.1	26.4	27.4	25.1	23.1	21.2	19.8	18.4	17.0			
Temperature	38.0	37.1	38.6	38.1	36.9	37.0	36.2	34.4	31.9			

Score 0 = normal

Laboratory mice are extremely susceptible to *E. rhusiopathiae* and clinical signs of the disease develop between one and 3 days after infection. Acute septicaemia causes death in unprotected mice within one or 2 days after the onset of clinical signs, and often animals are found dead unexpectedly. The only clinical signs were non-specific ones, such as ruffled fur, hunched posture or ocular discharge, and are common to many other bacterial infections like *E. coli* or listeriosis. There were no cardinal clinical signs specific for this infection. On the other hand, the test design creates partly-protected animals with vaccine dilutions. These animals could show signs of the disease and then either recover or reach the end of the assay in a chronic disease status. As the calculation of the test result is made in IU, the need to be confident that all animals would have died in the absence of an early intervention is essential.

Due to the rapid onset and short duration of the disease, measurement of body weight could not be used as a predictor for lethality as most animals died before a clear decrease in body weight was evident. Acute erysipelas disease in pigs is characterized by a rapid onset of high fever, but the mice did not show any hyperthermia. However, terminally

ill animals developed hypothermia, which is in accordance with the results of Soothill *et al.* (1992) with other bacterial infections.

In conclusion, for the immunization-challenge test of erysipelas vaccines it was not possible to define early predictors of lethality in mice and so, like Soothill *et al.* (1992), we recommend that terminally ill animals characterized by lethargy and body temperatures below 34°C should be humanely killed.

Recommendations for challenge tests in laboratory animals

- Wherever possible, humane endpoints based on clinical signs, body temperature and body weight should be used in batch potency challenge tests providing the scientific objective can still be achieved. Pharmacopoeias should introduce statements requiring such endpoints, to minimize causing avoidable pain and distress to the animals.
- Laboratory animals should be observed more than once a day, especially during the period of illness and potential implementation of humane endpoints.

- Where the use of humane endpoints is not feasible, terminally ill animals should be humanely killed and considered to have died from the disease.

Vaccination-challenge tests for veterinary vaccines in the target species

The efficacy of vaccines for veterinary use is primarily demonstrated by well-controlled experiments under laboratory conditions in which target animals are vaccinated and then challenged (see Castle 1999). Animal testing is required only for the licensing of a product and their specifications are included in the new or revised monographs of the *Ph.Eur.* (Council of Europe 1997). The challenge test attempts to mimic the natural conditions of infection as much as possible (Lensing *et al.* 1995) and only in very few cases are other assays allowed (e.g. serological tests for neutralizing and haemagglutinating antibodies for inactivated feline panleucopenia vaccine). The precise details of a test depend on the vaccine but many monographs do not usually provide for clinical signs to be used as humane endpoints, requiring only that vaccinated animals remain in good health whereas control animals die or display typical signs of infection (which may be quite severe). However, some recent monographs have detailed evaluation criteria including signs of disease, but no clear guidance is given for their use as humane endpoints. For example, the monographs on porcine actinobacillosis vaccine (inactivated) and on canine distemper vaccine state that to avoid unnecessary suffering, it is possible to kill severely ill animals or animals displaying typical signs, but the clinical signs indicating these states are not well defined. In contrast, the evaluation of efficacy of feline viral rhinotracheitis vaccine after intranasal challenge is based on a scoring system for body temperature, signs of disease and virus isolation from nasal washings. Surprisingly, and despite this, this test still uses death as a maximum score. For animal welfare reasons, death should not be scored higher than any other validated parameter that could equally

be used as a humane endpoint and retain the test's validity.

Recommendations for challenge tests in target animals

- All pharmacopoeial monographs should specify the typical clinical signs of the disease.
- Wherever possible, the body temperature should be recorded.
- Depending on the nature of the disease, all other realistic measurements should be used to differentiate between healthy and ill animals, e.g.
 - body weight;
 - re-isolation of challenge strains;
 - blood cell counts and other blood parameters; and
 - reduction in egg or milk production.
- Clear validated criteria for humane endpoints at the earliest possible stage should be stated in the monograph, and death should be positively discouraged as an acceptable endpoint.

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