Journal of Pharmacological and Toxicological Methods xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Appraisal of state-of-the-art

Study designs for the nonclinical safety testing of new vaccine products ☆

Roy Forster *

CiToxLAB France, P.O. Box 563, 27005 Evreux cedex, France

ARTICLE INFO

Article history:
Received 24 July 2011
Accepted 5 April 2012
Available online xxxx

Keywords:
Vaccine
Safety testing
Toxicology
Local tolerance
Safety pharmacology
Therapeutic vaccine

ABSTRACT

During the development of a new vaccine, the purpose of nonclinical studies is to provide safety information to support the clinical development and licensure of the product. In this article the study designs currently accepted for the nonclinical safety testing of new vaccines are described for single dose, local tolerance, repeat dose toxicity and safety pharmacology studies; these studies together form the basis of a typical nonclinical safety evaluation dossier. The detailed design of the preclinical package must take account of the intended clinical use, patient population, route of administration, formulation, dose level and immunisation schedule. The test item that is used for these studies must be adequately representative of the intended clinical formulation. The animal model used for these studies must be selected on criteria of relevance. Single dose toxicity studies provide information on acute actions or the potential effect of accidental overdose, but this information is often available from the repeat dose toxicity study, obviating the need for the acute study. Local tolerance studies provide information on tissue reactions at the site of administration. Evaluation of the findings must distinguish between normal tissue responses to injected material and findings indicative of undesirable pathological changes. The repeated dose toxicity studies are the principal studies that support the safety profile of the vaccines. The design of these studies must take full account of the features of the vaccine in the choice of treatment regime, dose levels, pharmacodynamic monitoring and timing of investigations and sacrifice. Safety pharmacology studies are performed to evaluate the potential for undesirable secondary pharmacological actions of vaccines if there is data to suggest that such studies are needed; this evaluation is made on a case by case basis. In the absence of specific guidance the design of studies for therapeutic vaccines follows the same general principles as those for anti-infective vaccines.

1. Introduction

The impact of vaccination on infectious diseases and public health in countries around the entire world has been enormous. Many previously dreaded infectious diseases such as smallpox, polio and tuberculosis or childhood diseases such as whooping cough, diphtheria, measles and mumps are now largely controlled by effective vaccination programmes. Progress is being made to develop vaccines against malaria and other parasitic and fungal diseases. And now, a new generation of "therapeutic" vaccines seems poised to provide novel therapeutic approaches to non-infectious chronic diseases such as cancer, asthma, diabetes, hypertension and others. Advances in our understanding of basic immunology have given greater insight into the working of vaccines, and building on this understanding we are living in a period of intense research and creativity in vaccine-based therapies.

Current vaccine products are a heterogeneous group and include inactivated bacterial and viral vaccines (cholera vaccine, influenza vaccine), live attenuated vaccine strains (such as measles vaccine), purified, recombinant or engineered proteins (e.g., Engerix hepatitis B vaccine), polysaccharide and conjugated vaccines (e.g., bacterial meningitis vaccine), DNA vaccines (e.g., Equine West Nile Virus vaccine) and (more recently) therapeutic vaccines still under development. Vaccine products also often contain adjuvants, which are also heterogeneous materials. Families of adjuvants include inorganic salts (such as alum), oil emulsions (e.g., MF59), lipid A fractions of LPS (e.g., MPL), saponin-based mixtures (e.g., QS-21) and oligonucleotides (e.g., CpG sequences).

Experience with vaccines over many decades has shown that vaccination is generally a safe and well-tolerated procedure. Nevertheless, toxic actions of vaccines can result from at least two sources: toxicity of constituent materials and toxicities linked to the pharmacodynamic action of the vaccine.

Toxicity resulting from constituent materials is the intrinsic (or "chemical") toxicity associated with injection into the recipient of a significant dose of exogenous material, often of microbial origin. This can result in local (or even systemic) toxicity and injection site reactions. This may be referred to as reactogenicity, a term used for

Abbreviations: BALT, bronchus associated lymphoid tissue; EMEA, EMA, European Medicines Agency; FDA, US Food and Drug Administration; FHD, full human dose; ICH, International Conference on Harmonisation on Technical Requirements for Registration of Pharmaceuticals for Human Use; LN, lymph node; LPS, lipopolysaccharide; NHP, non-human primates; WHO, World Health Organisation.

☆ Based on a paper presented at the Continuing Education Course (CEC-2), Regulatory toxicology and risk assessment processes for preventative and therapeutic vaccine development, IUTOX, Barcelona, July 2010.

* Tel.: +33 2 3229 2626; fax: +33 2 3262 8705.
E-mail address: roy.forster@citoxlab.com.

1056-8719/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.vascn.2012.04.003

the capacity of a vaccine to produce adverse reactions such as feverishness, sore arm, injection site induration or swelling.

The desired pharmacodynamic action of vaccines is the induction of a persistent immunological response against the targeted pathogen. The process of immunisation and the resultant immunological response can be the source of further potential toxicities. These toxicities may be seen as general systemic toxicity (fever, organ specific toxicity, malaise) or as immune-mediated toxicities such as anaphylaxis, cytokine release, immune suppression or autoimmune disease. In some cases more subtle immunological consequences may follow, such as paradoxical modification or enhancement of the target disease. These areas of potential toxicity were reviewed recently, illustrated with examples from clinical experience, by Goetz, Pfleiderer, & Schneider, 2010.

Live attenuated microbial strains can also present a risk associated with reversion to virulence (Offit, 2005). The mechanistic basis of attenuation of the vaccine strain may be well defined (as in the case of poliovirus) but for many vaccine strains is not fully understood. Molecular techniques are having an increased impact in virulence testing of production batches. This activity forms part of product quality control and does not fall in the domain of the nonclinical toxicologist.

What studies are required for the nonclinical safety studies to support the development of new vaccine products? The purpose of the nonclinical studies is to provide sufficient safety information to support the planned clinical development of the vaccine and subsequently to support licensure. The need for nonclinical safety studies and their relation to the conduct of human clinical trials is described in general terms in the International Conference on Harmonisation (ICH) guideline M3, and this document forms a useful basis for the nonclinical development program of vaccines; nevertheless vaccines are specifically excluded from the scope of some ICH documents (e.g., ICH guideline S6). At the time of writing, ICH drafting of "Guidelines for basic requirements for registration of vaccines" is in progress.

The current status of guidance documents for vaccines is reviewed in the accompanying article of Sun, Gruber, and Matsumoto (2012). The preclinical studies that are required for development of a vaccine are described in the EMEA (1997) Guideline on Nonclinical Studies for Vaccines and also in the WHO Guidelines on Nonclinical Evaluation of Vaccines (2003). The latter WHO guideline is recognised by both FDA and EMEA (now EMA). The conduct of nonclinical studies required for the development of new vaccines has recently been reviewed by Wolf, Kaplanski, and Lebron (2010), and the areas of regulatory debate surrounding these studies were discussed by van der Laan et al. (2009).

The present article describes the study designs currently accepted for the nonclinical safety testing of new vaccine products. This article does not deal with (i) the studies required reproductive toxicology studies that may be required, which are described in an accompanying article (Barrow, 2012), (ii) the preclinical safety studies required for the development of novel adjuvants, or (iii) the specific issues associated with DNA vaccines and experimental gene transfer.

The study designs are reviewed for single dose toxicity, local tolerance studies, repeat dose toxicity and safety pharmacology studies that together form the basis of a typical safety evaluation dossier supporting clinical trials and licensure. Traditional mutagenicity tests (such as the Ames test or mammalian cell-based assays) are not considered appropriate for new vaccine products; they are therefore generally not required and are not discussed here. In the same way, pharmacokinetics studies are generally not required (and are not discussed here), since the action of vaccines is mediated locally at the site of injection and draining lymph nodes.

2. General considerations and prerequisites

The purpose of the nonclinical studies is to support the intended clinical use of the vaccine product. For the design of a preclinical package, it will therefore be necessary to know some or all of the following: intended clinical use and patient population, route of administration and formulation, dose level and immunisation schedule. Armed with information of this kind the preclinical program can be designed to meet the needs of the clinical plan and support the proposed clinical studies.

The test item that is used for the preclinical studies must be adequately representative of the vaccine formulation that is intended for clinical use, and in particular all constituents intended to modulate the immunological response (adjuvants or other) must be present. There is no guideline or legislative obligation to use GMP grade material for the nonclinical studies, but the use of clinical batches or prevalidation batches will usually ensure the strict comparability of the vaccine used in nonclinical safety evaluation with clinical material and hence will justify the extrapolation of any findings. Placebo preparations containing excipient materials may be required for the treatment of control groups. If some kind of device will be used for administration of the vaccine in clinical use (e.g., nasal spray pump), then as far as possible the same device should be used in the animal study (allowing for scale and anatomical differences). If this cannot be achieved then a device giving similar performance should be used.

Administration in the toxicity studies must use the clinical route of administration. Traditional routes of administration for vaccination are by intramuscular, subcutaneous or intradermal routes. These routes of administration can readily be achieved in laboratory animals, but in rodents there are limitations on the greatest quantities that can be administered. In particular, while guidance documents emphasize the need to administer a full human dose (FHD) in safety studies, there may be problems to achieve the full volume used in humans. In part this can be overcome by the use of several sites of administration. Typical volumes (rather than maximal volumes) that can be achieved for these two routes are given in the text table below.

Typical values for administered volumes.

	Subcutaneous 1 site	Subcutaneous 4 sites	Intramuscular 1 site	Intramuscular 4 sites
Mouse (20 g)	0.5 ml	2 ml	0.05 ml	0.2 ml
Rat (200 g)	1 ml	4 ml	0.1 ml	0.4 ml
Rabbit (2.5 kg)	2 ml	8 ml	0.5 ml	2 ml

There is also current interest in "alternative" routes of administration for vaccines including mucosal routes (such as nasal, rectal or vaginal), oral and topical administration, and these routes can generally be achieved without difficulty in laboratory animals.

A repeated dose toxicity study in a single animal species is normally sufficient for vaccine products, and there is no requirement to perform studies in both rodent and nonrodent species. This is similar to requirements for biologics (such as monoclonal antibodies) where it is also sufficient that studies are performed in one relevant species, when supported by scientific justification. In contrast, for new "small molecule" medicines, nonclinical studies in two species (one rodent and one nonrodent) are required.

The selection of the animal model is a demanding question. Ideally, for vaccine safety studies, the selected species should fulfil several criteria:

- The selected species should develop an immune response following immunisation (humoral and/or cell mediated) that is similar to the expected response in humans after vaccination (so that toxicities related to the pharmacodynamic action of the vaccine can be identified).
- The selected species should demonstrate a similar immunological effect to any adjuvant used in the product (e.g., an adjuvant that directs a Th1 response in humans should direct the same response in the selected animal species).
- The selected species should be susceptible to the pathogen, reflecting the course of infection in man (and in the case of live attenuated vaccine strains, permitting evaluation of viremia/bacteremia).

Please cite this article as: Forster, R., Study designs for the nonclinical safety testing of new vaccine products , *Journal of Pharmacological and Toxicological Methods* (2012), doi:10.1016/j.vascn.2012.04.003

This is a demanding set of criteria, and in many cases there is no routine laboratory animal model that meets all three of these criteria. In addition, there are further purely practical factors that should also be considered in the selection of the animal model. Previous experience with the pathogen or family of pathogens in question, may provide useful guidance in selecting an appropriate model. Consideration should also be given to practical issues such as the feasibility of the proposed route of administration and volume to be given; availability of naïve animals with negative serology for the pathogen in question; and availability of reagents for immunological analyses.

Seen from an alternative perspective, where do the various laboratory animals that are commonly used in regulatory toxicology studies appear to be useful for vaccine studies? Rodents and rabbits are often used for vaccine safety studies (and are often also the species of choice for reproductive toxicity studies with the vaccine products, if required). The mouse can be a useful model where CTL responses must be characterised because of availability of reagents; some mouse strains also show a predisposition for either Th1 or Th2 responses (Darville et al., 2001). The rat is a well-characterised and versatile model in regulatory toxicology, for which there is a strong background of experience and of historical data. The rabbit is less often used than the rat in repeat-dose toxicology studies (excepting perhaps with dermally applied products) but usually demonstrates the required humoral response following administration of vaccines. For vaccine studies, the rabbit has the advantage that it is also sufficiently large that administration of the full human dose can generally be achieved, and the blood volume permits repeated blood samplings where needed. The minipig has occasionally been used for studies with human vaccines (Dincer, Jones, & Haworth, 2006; Glueck, 2001). The monkey is sometimes the most appropriate choice of species (Kennedy, Shearer, & Hildebrand, 1997) or the only species that meets criteria for selection. An example is given by measles vaccine; the macaque monkey is the only commonly used laboratory animal that is permissive for the measles virus, developing viremia after administration of the vaccine, and a measles-like disease on exposure to this virus (El Mubarak et al., 2007; Forster et al., 2008).

In some cases, less common laboratory animals may be appropriate. These include the ferret, the cotton rat (Niewiesk & Prince, 2002) and the hamster. The ferret is the best animal model for the pathophysiology of influenza, demonstrating human-like disease and transmission (EMEA ,2007), and may be used in general and reproductive toxicity studies. A drawback of such models is that hands-on experience in the manipulation of the animals and interpretation of findings may be limited and historical control data may be lacking. In such cases an alternative option that may deserve consideration is to perform GLP-compliant toxicology studies in an animal species that is routinely used for toxicology (e.g., rats and rabbits) and to perform non-GLP studies in the most indicated animal species to address specific safety issues (e.g., cotton rats, ferrets, etc.).

Specific models have been developed to address particular issues. In the case of dengue fever, exposure to the dengue virus does not result in infection or disease in normal rodents or rabbits while nonhuman primates show viremia but no disease. A transgenic mouse strain, lacking α- and β-interferon and gamma interferon receptor genes, proved susceptible to dengue virus. Administration of the virus to this DEN AG129 strain results in disease and lethality, and this model has been proposed for dengue vaccine development (Johnson & Roehrig, 1999). Transgenic mouse strains are available in which human MHC molecules are expressed permitting the study of human MHC restricted epitope sequences. HLA class I transgenic mice permit the study of cytotoxic T-lymphocyte responses to viral or tumoral epitopes that are MHC class I restricted (Pascolo, 2005). Transgenic mice expressing human MHC II molecules can permit the study of helper T-lymphocyte responses with MHC class II restricted epitopes. These transgenic models can play a role in the safety testing of vaccines based on human MHC restricted sequences (Depla et al., 2008).

Finally, there are some formal prerequisites concerning the performance of safety evaluation studies to support the development of new vaccines. It is an obligation that such studies are performed in compliance with relevant Good Laboratory Practice (GLP) regulations. For test items that are live (attenuated) viral or bacterial strains appropriate equipment and procedures will be required in order to guarantee confinement to meet the relevant national biosafety level (BSL) standard for the organism in question. This will generally imply approval by the Institutional Biosafety Committee of the facility where the study is performed. In addition, legislation may require that prior authorisations are obtained in order to work with pathogens or with genetically modified organisms (GMOs). Attention should be given to these questions before commencing any study.

3. Single dose toxicity study

Single dose (acute) toxicity studies on vaccine products can provide preliminary tolerability and safety data for a new vaccine and useful information in order to evaluate the acute actions of a vaccine. These studies are generally performed in rodents, and typically the study design will be that of a rodent acute toxicity study with administration of the FHD or greater.

The EMEA vaccine nonclinical guideline makes specific mention of single dose toxicity studies and states that such data should be available from at least one animal species, with a dose that provides an adequate margin of safety in relation with the intended human dose (EMEA, 1997). The guideline goes on to say that data of this kind may be equally obtained from other studies (such as immunogenicity studies or safety pharmacology studies). For many products, such data is available from the repeat-dose toxicity study (which monitors the same end-points of clinical signs, body weight and additional parameters such as clinical pathology). In consequence, separate single dose toxicity studies are often not performed when a repeat dose toxicity study will be available.

There may be circumstances, however, where a single dose toxicity study can be an important or even critical element of a nonclinical dossier. This may be the case where there is significant intrinsic toxicity of the antigen or of another component of the vaccine formulation; similarly, in some cases antigens (for example, under study for the development of therapeutic vaccines) may have marked pharmacological actions. Single dose studies may also be important where the immune response induced by the first administration significantly alters reactions to a second administration (e.g., neutralisation of viral vectors such as adenovirus constructs or neutralisation of “adjuvant” cytokines).

4. Local tolerance

Vaccines are typically administered by the subcutaneous, intradermal or intramuscular routes, and in clinical use it is not infrequent that administration is followed by local (injection site) reactions. The purpose of local tolerance studies is to evaluate tissue reactions at the site of administration by gross observation and by histopathology. Evaluation of sites inadvertently exposed to the vaccine may also be considered. If there are marked reactions, follow-up studies may examine the persistence of material (vaccine antigen and/or adjuvant) at the injection site and in draining lymph nodes. This evaluation can usually be made during the repeat-dose toxicity study with a new vaccine product since the repeated administrations (and hence numerous injection sites) usually provides ample opportunity for evaluation of local tolerance and the kinetics and resolution of any reaction. In some cases, however, a stand-alone study may be preferable or may provide an opportunity for more detailed investigations. For example, an evaluation of local tolerance in rabbits may be useful where the repeated dose toxicity study is performed in the mouse. An example of a study design of the evaluation of intramuscular local

Please cite this article as: Forster, R., Study designs for the nonclinical safety testing of new vaccine products , *Journal of Pharmacological and Toxicological Methods* (2012), doi:10.1016/j.vascn.2012.04.003

tolerance in rabbits is given in Table 1. Adjuvants very often produce local reactions, and it is therefore useful to include an "adjuvant-only" group in the study design, in order to evaluate the relative contribution of the adjuvant and vaccine components in producing local reactions.

Typical findings in such studies, depending on the severity of the tissue reactions, might include local reactions, pain, redness, swelling, granuloma formation, abscess, necrosis and regional lymphadenopathy. Fig. 1 shows an example of tissue responses at the intramuscular injection site due to alum adjuvant. The pathologist must take care to distinguish between normal tissue responses that can be considered healthy and vigorous responses to the injection of the test material (inflammatory responses, etc.) and findings that may be indicative of the development of undesirable pathological changes in the tissue (e.g., degenerative changes, giant cells, etc.).

C-reactive protein, an acute phase response protein involved in complement activation, is a sensitive marker of inflammatory changes in humans, nonhuman primates, dogs and rabbits. In clinical studies, elevated levels of c-reactive protein levels are found following immunisation with many vaccines that produce local reactogenicity, and C-reactive protein may therefore be a useful translatable biomarker to include in nonclinical local tolerance studies. If elevations in C-reactive protein are observed, the acute phase response may be confirmed by assessment of levels of other acute phase reactants using serum protein electrophoresis. In rat, however, C-reactive protein is not a good marker of inflammatory changes; there is some evidence that alpha-2-macroglobulin may be a useful alternative acute phase protein marker in the rat (Watterson, Lanevschi, Horner, & Louden, 2009).

5. Repeated dose (general toxicity) studies

The repeated dose toxicity studies are the principal studies that support the safety profile of the vaccines under development. The design of these studies is discussed in the WHO Guidelines on Nonclinical Evaluation of Vaccines (2003) and is broadly modelled on the repeat dose toxicity study design for medicinal products (as described, for example, in EMEA, 2010), but consideration must be given to vaccine specific issues in experimental choices regarding the treatment regime, selection of dose levels, pharmacodynamic monitoring, follow-up period and histopathology tissue list.

5.1. Treatment regime

The treatment regime used in the nonclinical study should follow the proposed clinical regime. For example, if the clinical regime consists of three immunizing administrations followed by booster administrations, the animal study should include three immunizing administrations followed by booster administrations. Since the intervals between vaccine administrations in clinical use may be very long, it is permissible in animal studies to compress the time plan, provided that the dosing interval is broadly consistent with the underlying immunological events. In particular, successive administrations in animals should be spaced at sufficient intervals that there is no interference between successive immunological responses. An interval of 2–3 weeks between successive administrations is considered to be sufficient (WHO Guidelines on Nonclinical Evaluation of Vaccines, 2003). In this way, a clinical immunisation regime with four administrations at successive intervals of 6 weeks, 6 months and 2 years would be simulated in animals by four administrations at intervals of 3 weeks.

Repeated administration of the vaccine product may result in an increasingly pronounced immune response. Accordingly, in order to provide confidence in the safety of the dosing schedule, the number of administrations in the toxicity study should exceed the number planned for human administration. This is generally referred to as the $(n+1)$ rule, which means that at least one more administration should be given than in the proposed clinical scheme.

Table 1
Example study design: intramuscular local tolerance study of a vaccine product in rabbit.

	Treatment	Group size
Group 1	Placebo; intramuscular route	3 M + 3 F
Group 2	Adjuvant control; intramuscular route	3 M + 3 F
Group 3	Vaccine (1 FHD); intramuscular route	3 M + 3 F

Clinical observations:

- Daily check
- Body weight prior to dosing and at day 3
- Examination of injection sites 3, 24, 48 and 72 h after inoculation; scoring of local reactions (erythema, eschar and oedema)
- Evaluate limb use impairment

Laboratory investigations:

- Serum fibrinogen and C-reactive protein; pre-dose and at 48 h

Post-mortem investigations:

- Macroscopic and microscopic (histopathology) examination of injection sites
- Macroscopic examination of thoracic and abdominal cavities
- Major organs collected (liver, kidneys, heart, lung and gonads) and preserved in formalin

5.2. Choice of dose level

The full human dose should be tested exactly as it will be given in the proposed clinical use, in the same formulation and at the same volume. This is indicated in the WHO guideline as follows: "one full human dose should be administered, not scaled for body weight or surface area, where feasible" Where this is not possible, the maximum feasible dose should be administered.

On occasions it may be desirable to include higher dose levels in the study, for example to evaluate the dose–response of a finding, or to provide some flexibility in the choice of the clinical dose. If so, then the higher dose levels should be only small multiples of the proposed clinical dose (e.g., 3-fold, 5-fold), in order to avoid any irrelevant immunological events (principally tolerisation) that may be triggered by very high multiples. Inclusion of lower dose levels may also be considered, and this may be useful particularly if unacceptable toxicities are observed and a NOAEL needs to be determined.

The study must also include appropriate control groups and consideration should be given to the need for placebo or vehicle groups, adjuvant-only groups or antigen-only groups, etc.

5.3. Pharmacodynamic monitoring

Monitoring of the pharmacodynamic response during the repeat dose toxicity study is a valuable element of the study for several reasons. First, demonstration of the pharmacodynamic response serves to confirm the relevance of the animal model that has been selected for the toxicity studies. Secondly, it permits identification of nonresponders. These nonresponder animals will be a less appropriate population for the evaluation of toxicity, and so consideration can be given to stratification of the study data according to responder/nonresponder status (where there is a known issue of nonresponders, it will be preferable to increase the group size so that an adequate number of responder animals is present in each study group). Finally, where adverse effects are found in the toxicity study, the pharmacodynamic data may permit a correlation of the toxic actions with the measured immune response.

Monitoring is generally achieved by determination of antibody levels in blood samples (typically by ELISA based methods) and/or cellular immunity (by the Elispot technique) and/or monitoring of a panel of systemic inflammatory cytokines (to characterize the response and systemic exposure).

Please cite this article as: Forster, R., Study designs for the nonclinical safety testing of new vaccine products , *Journal of Pharmacological and Toxicological Methods* (2012), doi:10.1016/j.vascn.2012.04.003

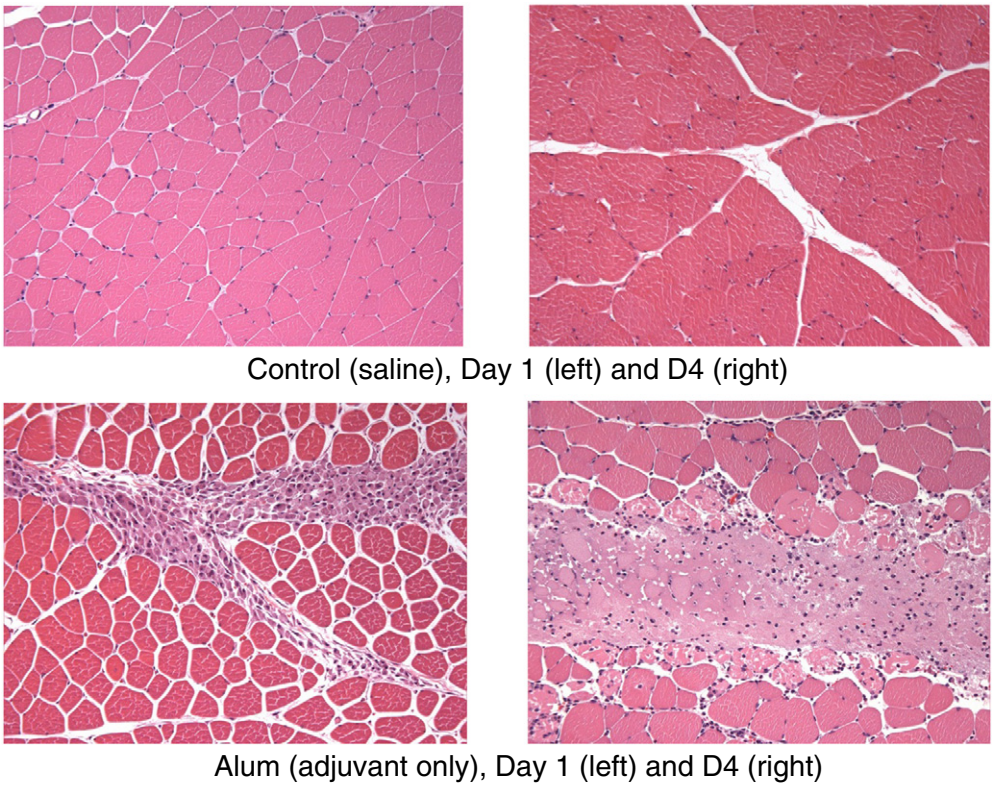

Fig. 1. Local tolerance: tissue reactions following intramuscular injection of alum adjuvant.Control skeletal muscle injected with saline has a normal histological aspect, and there is no detectable inflammatory response at 1 or 4 days. Following injection of alum, mononuclear inflammatory cell infiltrates (consisting of macrophages with violet cytoplasm) can be seen in the muscle perimysium as soon as 24 h after injection. After 4 days, necrotic material can be seen along with the mononuclear cell infiltrate.

5.4. Follow-up period

The repeat dose study must evaluate potential toxicities arising from immediate events involved in immunisation, physiological responses, reactogenicity and (for live vaccines) attenuated pathogenicity. For these events, in vivo (e.g., clinical pathology) and post mortem (e.g., histopathology) investigations need to be made at an early time point, when these events and responses are expected to be at peak levels.

The study must also evaluate any potential toxicities that arise from the immune response and any persistent effects. For this reason, a second series of in vivo and post mortem investigations are required at a late time-point when an evaluation of the persistence, exacerbation and/or recovery from any adverse effects is possible.

For each of these evaluations ("early" and "late"), the group size must be sufficient for the detection of adverse effects. Hence in vaccine studies with early and late sacrifice time the overall number of animals is generally doubled to ensure adequate animal numbers at each sacrifice time.

5.5. Tissue list

Histopathology is usually performed on a full range of tissues (as defined, for example, in EMEA, 2010). Consideration can be given to examination of a reduced tissue list for studies on vaccines with a well established record of safety and/or clinical safety (an example is given by the testing of annual strains of influenza vaccine). The WHO guideline indicates that the tissue list can be defined on a case-by-case basis following consultation with regulatory authorities. Nevertheless, a reduced tissue list for histopathological evaluation must include

- organs or tissues that are concerned by the selected route of administration,
- pivotal organs (brain, kidney, liver, gonads), and
- special attention to the immune organs (local and remote LNs, thymus, spleen, bone marrow, Peyer's patches, BALT, etc.).

In vivo evaluations should include ophthalmological evaluations, since uveitis may be indicative of autoimmune responses.

5.6. Overall

An example of a study design that brings together these elements is given in Table 2, which shows a study design for a two-administration rat toxicity study intended to support the single administration of a viral vaccine product in humans. Evaluation of systemic exposure (toxicokinetic evaluation) is not generally required for vaccines.

6. Safety pharmacology

Safety pharmacology studies are performed to evaluate the potential for undesirable secondary pharmacological actions of vaccines. Any pharmacological actions that affect vital physiological functions such as respiratory and cardiovascular function or the central nervous system may be of particular concern. Accordingly if there is data to suggest that the vaccine may affect physiological functions other than the immune system, safety pharmacology studies should be incorporated into the toxicity assessment. In the absence of such data, these studies need not routinely included in safety evaluation dossiers for new vaccine products. Hence an evaluation of the need for these studies can be made on a product-specific basis. An example of such effects is given by *Bordetella pertussis* (whooping cough) vaccine, which induces changes in diastolic blood pressure, heart rate and autonomic responsiveness in rats after a small number of repeated administrations (van Amsterdam et al., 1998).

Please cite this article as: Forster, R., Study designs for the nonclinical safety testing of new vaccine products , *Journal of Pharmacological and Toxicological Methods* (2012), doi:10.1016/j.vascn.2012.04.003

Table 2
Viral vaccine — 2 dose toxicity study in rats.

	Treatment	Group size	Early sacrifice	Late sacrifice
Group 1	Placebo	20 M + 20 F	10 M + 10 F	10 M + 10 F
Group 2	Adjuvant control	20 M + 20 F	10 M + 10 F	10 M + 10 F
Group 3	Vaccine product 1 FHD	20 M + 20 F	10 M + 10 F	10 M + 10 F
Group 4	Vaccine product 5 FHD	20 M + 20 F	10 M + 10 F	10 M + 10 F

Schedule

- Day 1: First administration
- Day 7: Hematology, blood biochemistry, urinalysis
- Day 7: Early sacrifice
- Day 18: Hematology, blood biochemistry, urinalysis
- Day 21: Second administration
- Day 28: (Optional investigations)
- Day 42: Hematology, blood biochemistry, urinalysis
- Day 42: Late sacrifice

Clinical observations

- Cageside observations, body weight and food consumption
- Body temperature (e.g., 6 h, 24 h after administration)
- Ophthalmology

Laboratory investigations

- Hematology, blood biochemistry, urinalysis
- Inflammation biomarker: alpha-2-macroglobulin at day 2/3 and day 7
- Viral burden in blood
- Antibody levels

Post-mortem examinations

- Macroscopic findings and organ weights
- Histopathology (full list of tissues, attention to lymphoid organs, site of administration, target organs for disease, etc.)

Study designs may be adapted to the characteristics of the vaccine test item. For example, Brennan and Dougan (2005) suggest a combined cardiovascular and respiratory study in rats. The test item is administered as a single dose by the clinical route and using the final formulation of the vaccine product. A "worst case" treatment group may receive unadjuvated antigen only by the intravenous route to simulate escape to the systemic circulation of the vaccine antigen. Cardiovascular (ECG, heart rate, blood pressure; by telemetry) and respiratory parameters (rate and volume; by plethysmography) should be monitored at early and late time-points. The early time-point (e.g., some hours after administration) is intended to assess direct toxicity, mediated by circulating toxins or cytokine release while the late time-point (e.g., some days after administration) is intended to assess effects mediated by the immune response.

In large animals, monitoring of parameters such as body temperature, electrocardiogram and CNS can be incorporated into the repeat dose toxicity studies. There is increasing interest in "integrated" evaluation of safety pharmacology in this way, and this can be readily achieved in large animal studies using external telemetry approaches.

Where a standalone study is required, this will resemble safety pharmacology studies routinely performed with candidate medicines. An example study design in given in Table 3.

In vitro evaluation of the potential for QT prolongation (for example, with the hERG assay) is not usually required for vaccine products; QT prolongation can be evaluated in vivo in telemetry studies as described above.

7. Therapeutic vaccines

There is currently great interest in the potential of therapeutic vaccines, which use the patient's immune system to combat diseases such cancer, asthma, Alzheimer's disease, hypertension or diabetes. The target population therefore consists of patients, unlike prophylactic vaccination for infectious disease, where the target population consists of healthy subjects, in many cases children.

Therapeutic vaccines are specifically excluded from the scope of EMEA (1997), WHO Guidelines on Nonclinical Evaluation of Vaccines (2003) and ICH S6 guidance documents. On the other hand, the EMEA Guideline on Adjuvants in Vacines for Human Use (EMEA, 2005) states: "The principles of this guideline should also be applicable to quality and nonclinical aspects of 'therapeutic vaccines' (e.g., 'tumour vaccines')." A draft FDA guidance document on "Clinical Considerations for Therapeutic Cancer Vaccines" provides a few paragraphs of discussion of the preclinical testing requirements (FDA/CBER, 2009), principally dealing with the relationship between clinical and preclinical dose-levels and dosing regime.

In the absence of specific guidance, the design of studies for therapeutic vaccines has been inspired by the same principles as those applied to prophylactic (anti-infectious) vaccines. In many cases, however, these vaccines are intended for the treatment of chronic disease by repeated administration at intervals (for example, 1 or 3 months). In these cases, the $(n+1)$ rule does not appear to be entirely appropriate. Consideration must be given to the appropriate duration of a chronic (repeat dose) toxicity and how many cycles of administration are required before no further useful safety data will be generated (a similar issue may be faced in the evaluation of some gene therapy vectors where there is long lasting expression). In many cases, sponsors who are actively developing therapeutic vaccines will be also seeking advice directly from regulatory bodies, and can obtain direct feedback on questions of this kind. By analogy to ICH Guideline S6(R1) (ICH, 2011), there is probably a case for limiting the duration of such studies to 6 months.

8. Conclusions

This brief review of the nonclinical safety testing of vaccines demonstrates that, in order to ensure careful and thorough testing, full account must be taken of the characteristics of the vaccines and of the underlying biology of the immune response that they provoke. The increasing sophistication of vaccines and therapies based on manipulation of the immune system will continue to raise issues and interesting questions for the preclinical toxicologist.

Table 3
Example study design: cardiovascular and respiratory safety pharmacology study in the beagle dog.

Animal species/strain	Telemetered Beagle dog (or other species, to be justified)
Number of animals	4 dogs/group; single group treated in cross-over design
Administration	Single administration by intended clinical route (e.g., intramuscular)
Dose level	1 full human dose (FHD)
Clinical observations	Twice daily
Body weight	At least weekly
Body temperature	Continuously recorded during the whole experiment
Cardiovascular parameters	Continuously recorded during the whole experiment- Mean arterial pressure, systolic and diastolic pressure - Heart rate- ECG parameters: QRS, PR, RR and QT intervals (ms)
Respiratory parameters	Continuously recorded during the whole experiment : - Respiration rate (f, breath/min),- Inspiratory time (TI, ms),- Expiratory time (TE, ms).
Data analysis	Data collected and averaged at selected time-points before treatment (−60, −40, −20 min) and after treatment (1, 3, 6, 24, 48 and 72 h). Duration can be extended on case-by-case basis.

Please cite this article as: Forster, R., Study designs for the nonclinical safety testing of new vaccine products , *Journal of Pharmacological and Toxicological Methods* (2012), doi:10.1016/j.vascn.2012.04.003

Acknowledgements

The author thanks Dr Lawrence Segal (GSK Biologicals, Belgium) and Sarah Gould (Sanofi Pasteur, France) for their helpful comments during the preparation of the material presented in this article.

References

Barrow, P. (2012, March–April). Developmental and reproductive toxicity testing of vaccines. *Journal of Pharmacological and Toxicological Methods*, *65*(2), 58–63.

Brennan, F. R., & Dougan, G. (2005). Nonclinical safety evaluation of novel vaccines and adjuvants: new products, new strategies. *Vaccine*, *23*, 3210–3222.

Darville, T., Andrews, C. W., Sikes, J. D., Fraley, P. L., Braswell, L., & Rank, R. G. (2001). Mouse strain-dependent chemokine regulation of the genital tract T helper cell type 1 immune response. *Infection and Immunity*, *69*, 7419–7424.

Depla, E., Van der Aa, A., Livingston, B. D., Crimi, C., Allosery, K., De Brabandere, V., et al. (2008). Rational design of a multiepitope vaccine encoding T-lymphocyte epitopes for treatment of chronic hepatitis B virus infections. *Journal of Virology*, *82*(1), 435–450.

Dincer, Z., Jones, S., & Haworth, R. (2006). Preclinical safety assessment of a DNA vaccine using particle-mediated epidermal delivery in domestic pig, minipig and mouse. *Experimental and Toxicologic Pathology*, *57*, 351–357.

El Mubarak, H. S., Yuksel, S., van Amerongen, G., Mulder, P. G. H., Mukhtar, M. M., Osterhaus, A. D. M. E., et al. (2007). Infection of cynomolgus macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) with different wild-type measles viruses. *Journal of General Virology*, *88*, 2028–2034.

EMEA. (1997). *Note for guidance on preclinical pharmacological and toxicological testing of vaccines, CPMP/SWP/465/95.*

EMEA. (2005). *Guideline on adjuvants in vaccines for human use, CHMP/VEG/134716/2004.*

EMEA. (2007). *Guideline on influenza vaccines prepared from viruses with the potential to cause a pandemic and intended for use outside of the core dossier content, EMEA/CHMP/VWP/263499/2006.*

EMEA. (2010). *Guideline on repeated dose toxicity, CPMP/SWP/1042/99 Rev 1 Corr*.*

FDA/CBER (2009). *Guidance for industry, clinical considerations for therapeutic cancer vaccines, draft guidance, FDA/CBER.*

Forster, R., Appelqvist, T., Brown, D., Cohen, B., Chaloner-Larsson, G., de Swart, R., et al. (2008). Pulmonary delivery of measles vaccine: Non-human primate safety study. *The Toxicologist*, *102*(1) Abstract No. 2297.

Glueck, R. (2001). Pre-clinical and clinical investigation of the safety of a novel adjuvant for intranasal immunization. *Vaccine*, *15*(Suppl. 1), S42–S44.

Goetz, K. B., Pfleiderer, M., & Schneider, C. K. (2010). First-in-human clinical trials with vaccines–what regulators want. *Nature Biotechnology*, *28*(9), 910–916.

ICH. (2011). *Preclinical safety evaluation of biotechnology derived pharmaceuticals, ICH S6(R1), June 2011.*

Johnson, A. J., & Roehrig, J. T. (1999). New mouse model for dengue virus vaccine testing. *Journal of Virology*, *73*(1), 783–786.

Kennedy, R. C., Shearer, M. H., & Hildebrand, W. (1997). Nonhuman primate models to evaluate vaccine safety and immunogenicity. *Vaccine*, *15*, 903–908.

Niewiesk, S., & Prince, G. (2002). Diversifying animal models: the use of hispid cotton rats (*Sigmodon hispidus*) in infectious diseases. *Laboratory Animals*, *36*, 357–372.

Offit, P. A. (2007). *The Cutter incident: How America's first polio vaccine led to the growing vaccine crisis.* New Haven, USA: Yale University Press.

Pascolo, S. (2005). HLA class I transgenic mice: development, utilisation and improvement. *Expert Opinion on Biological Therapy*, *5*(7), 919–938.

Sun, Yuansheng, Gruber, Marion, & Matsumoto, Mineo (2012, March–April). Overview of global regulatory toxicology requirements for vaccines and adjuvants. *Journal of Pharmacological and Toxicological Methods*, *65*(2), 49–57.

van der Laan, J. W., Forster, R., Ledwith, B., Gruber, M., Gould, S., Segal, L., et al. (2009). Nonclinical testing of vaccines: Report from a workshop. *Drug Information Journal*, *43*, 97–107.

van Amsterdam, J. G., te Biesebeek, J. D., van de Kuil, T., van der Laan, J. W., de Wildt, D. J., & Vleeming, W. (1998). Repeated administration of whole-cell and acellular pertussis vaccines affects haemodynamics and autonomic responsiveness. *Vaccine*, *16*(17), 1668–1674.

Watterson, C., Lanevschi, A., Horner, J., & Louden, C. (2009). A Comparative analysis of acute-phase proteins as inflammatory biomarkers in preclinical toxicology studies: Implications for preclinical to clinical translation. *Toxicologic Pathology*, *37*, 28–33.

WHO Guidelines on Nonclinical Evaluation of Vaccines. (2003).. Geneva, Switzerland: WHO.

Wolf, J. J., Kaplanski, C. V., & Lebron, J. A. (2010). Nonclinical safety assessment of vaccines and adjuvants. In G. Davies (Ed.), *Vaccine adjuvants, methods in molecular biology* (pp. 29–40). New York: Humana Press.

Please cite this article as: Forster, R., Study designs for the nonclinical safety testing of new vaccine products , *Journal of Pharmacological and Toxicological Methods* (2012), doi:10.1016/j.vascn.2012.04.003