

Annex 1

WHO guidelines on nonclinical evaluation of vaccines

This document provides guidance to national regulatory authorities (NRAs) and vaccine manufacturers on the nonclinical evaluation of vaccines by outlining the international regulatory expectations in this area. It should be read in conjunction with the Guidelines on clinical evaluation of vaccines: regulatory expectations (1), in order to complete the understanding of the whole process of vaccine evaluation. Vaccines are a diverse class of biological products and their nonclinical testing programmes will depend on product-specific features and clinical indications. The following text has therefore been written in the form of guidelines rather than recommendations. Guidelines allow greater flexibility than recommendations with respect to specific issues related to particular vaccines.

Introduction	32
1 General remarks	32
1.1 Scope	34
1.2 Glossary	34
2 Characterization of candidate vaccines	37
3 Immunogenicity and other pharmacodynamic studies	43
4 Toxicity assessment	44
4.1 Basic toxicity assessment	45
4.2 Additional toxicity assessments	48
5 Special considerations	51
5.1 Adjuvants	51
5.2 Additives (excipients and preservatives)	52
5.3 Vaccine formulation and delivery device	52
5.4 Alternative routes of administration	53
6 Specific considerations for particular types of vaccines	54
Authors	56
References	58
Appendix	
List of tissues to be collected in a repeated dose toxicity study	62

Introduction

Recent progress in biotechnology and basic immunology has led to the development of a broad range of novel vaccines raising exciting possibilities for the prevention of infectious diseases (2, 3). Improvements to already licensed vaccines are also being considered; such improvements will lead to new products as well as to the introduction of new adjuvants. However, the complexity and novelty of these products presents scientific and regulatory challenges because criteria for their safety, potency and quality assessment may not exist. Product diversity and new approaches, technologies and methodologies develop over time; therefore, judgement based on the best science available should always form the basis for deciding on the type and extent of nonclinical evaluation for these products.

Although nonclinical evaluation plays an essential part in the overall development of vaccine candidates, there is at present limited guidance regarding nonclinical evaluation programmes for these products. In this guidance document, the general principles of nonclinical evaluation of vaccines are discussed, with particular attention being given to the regulatory expectations for new and novel vaccines.

Preclinical testing is a prerequisite to moving a candidate vaccine from the laboratory to the clinic and includes all aspects of testing, product characterization, proof of concept/immunogenicity studies and safety testing in animals conducted prior to clinical testing of the product in humans. Nonclinical evaluation, within the context of this document, refers to all *in vivo* and *in vitro* testing performed before and during the clinical development of vaccines. For example, nonclinical evaluation may be necessary when changes in the manufacturing process or product formulations are made or to further study potential safety concerns that may have arisen from phase I and II trials or that have been described in the literature for similar products.

1 General remarks

Nonclinical studies are aimed at defining the *in vitro* and *in vivo* characteristics of candidate vaccines including those relating to safety and immunogenicity. Nonclinical studies in animals are valuable tools for identifying possible risks to the vaccinees and helping to plan protocols for subsequent clinical studies in human subjects. However, in all cases, when safety testing in animals is performed, there should be a clear rationale for doing so and the study should be performed in

compliance with the national and international laws for the protection of laboratory animals (4), biosafety requirements (5) and with good laboratory practice (GLP) (6). However, there may be situations where full compliance with GLP is not possible. If the study, or part of the study, was not conducted in compliance with GLP, areas of noncompliance should be defined and a statement of the reason for noncompliance should be drawn up.

Potential safety concerns for a vaccine product include those due to inherent toxicities of the product, toxicities of impurities and contaminants, and toxicities that result from interactions between the vaccine components present in the vaccine formulation. In addition, the immune response induced by the vaccine may lead to toxic side-effects.

Despite efforts to maximize the predictive value of nonclinical toxicity studies there is always the possibility that not all risks are identified. The limitations of animal testing in reflecting clinical safety and efficacy in humans should be recognized as pathogenesis and immune responses are frequently species-specific. Moreover, potential safety concerns identified during animal testing may not necessarily indicate a problem in humans. However, any signal observed in nonclinical toxicity studies should be carefully addressed in human clinical trials and may require additional nonclinical testing. It should be noted that the absence of detectable toxicity in animal studies does not necessarily mean a vaccine will be safe in humans. Potential safety concerns related to specific types of vaccine candidate are considered in section 6.

The development and subsequent validation of *in vitro* tests for use as alternatives to nonclinical evaluation of vaccine candidates in animals is encouraged as it may lead to the improvement of nonclinical testing as well as to a reduction of animal usage.

The need for and extent of nonclinical testing will depend on the product under consideration. For example, for a product for which there is no prior nonclinical and clinical experience, nonclinical testing would be expected to be more extensive than for those vaccines previously licensed and used in humans. In some cases, it may not be necessary to perform preclinical safety studies prior to the initiation of phase 1 clinical trials. For example, in the case of transfer of technology, where access to the database of the originally developed vaccine is available, data from nonclinical bridging studies (e.g. physicochemical characterization and abbreviated *in vivo* studies) may be an acceptable basis for further development of the product.

Early communication between the vaccine manufacturer and the responsible national regulatory authority to agree on the requirements for and type of nonclinical testing is recommended.

1.1 **Scope**

For the purposes of this document, vaccines are considered to be a heterogeneous class of medicinal products containing immunogenic substances capable of inducing specific, active and protective host immunity against infectious disease.

Although most vaccines are being developed for pre- and post-exposure prophylaxis, in some cases, they may be indicated for therapeutic use against infectious diseases, e.g. human immunodeficiency virus (HIV), and human papillomavirus (HPV). Both prophylactic and therapeutic vaccines for infectious disease indications are considered in this document.

Vaccines for human use include one or more of the following: microorganisms inactivated by chemical and/or physical means that retain appropriate immunogenic properties; living microorganisms that have been selected for their attenuation whilst retaining immunogenic properties; antigens extracted from microorganisms, secreted by them or produced by recombinant DNA technology; chimeric microorganisms; antigens produced in vivo in the vaccinated host following administration of a live vector or nucleic acid or antigens produced by chemical synthesis in vitro. The antigens may be in their native state, truncated or modified following introduction of mutations, detoxified by chemical or physical means and/or aggregated, polymerized or conjugated to a carrier to increase immunogenicity. Antigens may be presented plain or in conjunction with an adjuvant, or in combination with other antigens, additives and other excipients.

Therapeutic vaccines for non-infectious diseases (e.g. certain cancer vaccines) and monoclonal antibodies used as immunogens (e.g. anti-idiotypic antibodies) are *not* considered here.

1.2 **Glossary**

The definitions given below apply to the terms used in these guidelines. They may have different meanings in other contexts.

Adjuvants

Substances that are intended to enhance relevant immune response and subsequent clinical efficacy of the vaccine.

Booster vaccination

Vaccination given at a certain time interval after primary vaccination to enhance immune responses and induce long-term protection.

Combination vaccine

A vaccine that consists of two or more antigens, either combined by the manufacturer or mixed immediately before administration and intended to protect against either more than one disease, or against one disease caused by different strains or serotypes of the same organism.

Genetically modified organism (GMO)

An organism or a microorganism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. This definition covers microorganisms including viruses, viroids and cell cultures including those from animals, but does not cover naked recombinant DNA or naked recombinant plasmids.

Good clinical practice (GCP)

A standard for clinical studies that encompasses their design, conduct, monitoring, termination, audit, analyses, reporting and documentation and which ensures that the studies are scientifically and ethically sound and that the clinical properties (diagnostic, therapeutic or prophylactic) of the pharmaceutical product under investigation are properly documented.

Good laboratory practice (GLP)

A quality system concerned with the organizational process and the conditions under which nonclinical health and environmental safety studies are planned, performed, monitored, recorded, archived and reported. GLP principles may be considered as a set of criteria to be satisfied as a basis for ensuring the quality, reliability and integrity of studies, the reporting of verifiable conclusions and the traceability of data.

Good manufacturing practice (GMP)

A part of the pharmaceutical quality assurance which ensures that products are consistently produced and controlled according to the quality standards appropriate to their intended use and as required by the marketing authorization. In these guidelines, GMP refers to the current GMP guidelines published by WHO.

Immunogenicity

Capacity of a vaccine to induce antibody-mediated and/or cell-mediated immunity and/or immunological memory.

Nonclinical evaluation of vaccines

All in vivo and in vitro testing performed before and during clinical development of vaccines. The potential toxicity of a vaccine should be assessed not only prior to initiation of human trials, but throughout clinical development.

Plasmid

Double-stranded circular DNA molecules capable of replicating in bacterial cells.

Potency

The measure of biological activity, using a suitable quantitative biological assay, based on the attribute of the product that is linked to the relevant biological properties.

Preclinical evaluation of vaccine

All in vivo and in vitro testing carried out prior to the first testing of vaccines in humans. This is a prerequisite to the initiation of clinical trials and includes product characterization, proof of concept/immunogenicity studies and animal safety testing.

Preclinical toxicity study

A study designed with the primary purpose of demonstrating the safety and tolerability of a candidate vaccine product. The design of the preclinical toxicity study should meet the criteria outlined in the section on study design to be considered supportive of the intended clinical trial.

Primary vaccination

First vaccination or series of vaccinations given within a predefined period, with an interval of less than 6 months between doses, to induce clinical protection.

Product characterization

A full battery of physical, chemical and biological tests conducted for a particular product. These tests include, but are not limited to, in-process control testing, testing for adventitious agents, testing process additives and process intermediates, and lot release.

Protocol or study plan

A document that states the background, rationale and objectives of the nonclinical studies and describes its design, methodology and organization, including statistical considerations, and the conditions under which it is to be performed and managed.

Relevant animal model

An animal that develops an immune response similar to the expected human response after vaccination. It is acknowledged that species-specific differences in immune responses are likely. Ideally, the animal species chosen should be sensitive to the pathogenic organism or toxin under consideration.

Route of administration

The means by which the candidate vaccine product is introduced to the host. Possible routes of administration include the intravenous, intramuscular, subcutaneous, transcutaneous, intradermal, transdermal, oral, intranasal, intranodal, intravaginal and intrarectal routes.

Seroconversion

Predefined increase in antibody concentration, considered to correlate with the transition from seronegative to seropositive, providing information on the immunogenicity of a vaccine. If there are pre-existing antibodies, seroconversion is defined as a transition from a predefined low level to a significantly higher defined level, such as a fourfold increase in geometric mean antibody concentration.

Validation

The action of proving, in accordance with the principles of good manufacturing practice, that any procedure, process, equipment (including the computer software or hardware used), material, activity or system actually leads to the expected results.

2 Characterization of candidate vaccines

2.1 Vaccine production

The biological nature of the starting materials, the manufacturing process and the test methods needed to characterize batches of the product are important elements to be considered in the design and the interpretation of nonclinical testing of vaccines. Many vaccines are produced using prokaryotic or eukaryotic microorganisms and subtle changes in these organisms may radically affect the vaccine product. Therefore, the establishment of a seed-lot system is essential for vaccine production. Moreover, the quality, safety and potency of

these products are usually sensitive to changes in manufacturing conditions. The quality and safety of vaccine preparations cannot be assured solely by testing of the end-product, but depends on the strict control of the manufacturing process following the principles of good manufacturing practice (GMP) (7). This includes demonstration of the purity and quality of the starting material (raw materials and seeds), in-process control testing, testing for process additives and process intermediates and the development and establishment of lot release tests. Moreover, as the relationship between physical and chemical characteristics, and the immunogenicity and efficacy of these products is frequently not completely understood, biological characterization through the use of biological assays should always complement the physical and chemical product characterization. The development of appropriate laboratory methods to characterize a vaccine formulation with respect to its components, as well as its safety and potency, is a prerequisite to the clinical use of any new or novel vaccines against bacteria, viruses or parasites.

Consistency of production is essential, and the demonstration that the product does not differ from vaccine lots that have been shown to be safe and adequately immunogenic and protective in clinical studies is a crucial component of vaccine evaluation, licensing and batch release. For this reason, manufacturers should make every effort to characterize these clinical lots and if possible to keep some of these lots for future reference.

Where no appropriate animal model exists for testing potency or where direct serological or immunological correlates of clinical protection are not available, the challenge is to ensure that each production batch has the same protective efficacy as those batches shown to be protective in clinical trials. In such cases, emphasis is increasingly being placed on assuring the consistency of production using modern physical, chemical and immunological methods that enable characterization of some products to a degree of precision not previously possible.

The vaccine lots used in preclinical studies should be adequately representative of the formulation intended for use in the clinical investigation and, ideally, preclinical testing should be done on the same lot as that proposed for the clinical trials. If this is not feasible, then the lots studied should be comparable with respect to physico-chemical data, stability and formulation.

At a minimum, candidate vaccines for clinical trials should be prepared under conditions of good manufacturing practice (GMP) for

clinical trial material (8). However full GMP will be required at the later stages of clinical development (7, 9).

Any change proposed to the manufacturing process during vaccine development should be considered carefully to evaluate its impact on the quality, safety and efficacy of the vaccine and the possible need for additional nonclinical and clinical investigations.

Subsequent changes in production methods or scale-up following product licensure will necessitate further product characterization to demonstrate comparability with the original lot(s) used to demonstrate safety and efficacy of the product. The extent of comparability testing needed depends on the nature of the changes implemented (10). These changes should be documented and the national regulatory authority consulted. Regulatory authorities should clearly define and implement in their regulations what changes require only a notification and which changes require formal approval before implementation (11).

The procedures used in the characterization and control of existing licensed traditional vaccines are not likely to be applicable to newer products developed using state-of-the-art technology to protect against the same infection. For example, specific guidelines have been developed for the production and control of acellular pertussis vaccines that differ from those applied to whole cell pertussis vaccine (12). Likewise, the tests applied to the characterization and control of traditional inactivated cholera vaccine for parenteral use are not necessarily applicable to the new inactivated whole-cell cholera vaccine intended for oral administration, and an appropriate potency test for the oral vaccine needs to be developed.

2.2 **Potency**

Potency tests measure the biological activity of a vaccine but do not necessarily reflect the mechanism of protection in humans. Potency measurement is often used to verify the consistency of the manufacturing process. The initial concept of potency testing for vaccines was to quantify the biological activity of the vaccine in comparison with a reference preparation of known bioactivity, where the antigenic component(s) were not well-defined.

Classical challenge studies in animals immunized with the vaccine under consideration have been developed into routine potency assays (e.g. for diphtheria and tetanus toxoids). In the case of the whole-cell pertussis potency assay, which consists of intracerebral challenge of immunized and nonimmunized animals, a correlation was established with clinical protection in humans (11). Where no suitable animal

challenge model exists, potency is often based on measurement of immune responses, usually serological (e.g. influenza and hepatitis B vaccines).

More recently, recombinant DNA methodology and modern physico-chemical techniques have resulted in the manufacture of highly purified products that can be better characterized than the classic biologicals. However, the ability to measure the “relevant” biological activity for such products may still be lacking. For these products, characterization using physicochemical parameters, such as amount of antigen, size of the antigen, protein content and others can be used as a measure of consistency, but not necessarily of the potency of a vaccine.

For live attenuated vaccines, the approach to potency measurement is generally different. The potency of live viral vaccines is usually based on titration of the minimum infective dose in cell culture or chicken embryos, which may be considered as a surrogate marker of potency, but not as a measure of potency itself. A similar approach is taken to the potency measurement of live attenuated bacterial vaccines, bacille Calmette–Guérin (BCG), and typhoid vaccine (live Ty21A oral), where the number of live organisms present is the measure of potency.

For vaccines that express inserts encoding heterologous vaccine antigens (vaccines based on viral or bacterial vectors), it is not sufficient to determine the “biological activity” of the entire construct by measuring colony forming units (CFU) or infectious titre. For these vaccines, the use of other methods such as the quantitation of the expression of the insert, or the evaluation of the effective dose (ED_{50}) of the vectored vaccine should be considered.

2.3 **Stability**

The evaluation of vaccine stability is complex, as they are very susceptible to inactivation by environmental factors. Potency, as defined in the glossary, should be measured as a part of the stability testing, except in those cases where potency testing based on biological activity is not possible. Physical and chemical product characterization should be included in the stability evaluation. For a product entering human clinical trials, sufficient data should be collected to support the stability of the product for the duration of the preclinical and clinical trial. In certain cases, accelerated stability data may be used to support preliminary data obtained at the normal storage temperature. Stability data to support licensure should be obtained under the proposed storage conditions and should be based on long-term,

real-time stability studies. Finally, the stability of standards and reference materials also needs to be considered to ensure that the procedures used to measure relevant parameters are reliably standardized.

2.4 ***International and national guidelines***

The World Health Organization (WHO), through considerable international consultation, develops Recommendations and Guidelines on the production and control of vaccines and other important biologicals (13), and these form the basis for assuring the acceptability of products globally. These documents specify the need for appropriate starting materials, including seed lot system and cell banks; strict adherence to established protocols; tests for purity, potency, and safety at specific steps during production; and the keeping of proper records. Guidelines allow greater flexibility than Recommendations with respect to specific issues related to particular vaccines.

WHO also provides Guidelines on manufacturing establishments involved in vaccine production. Recommendations can be found in the WHO document on good manufacturing practice for biologicals (7). Particular attention should be given to developing documented standard operating procedures for both production processes and testing procedures. These should be introduced as early as possible during the development of a vaccine and be well established by the time phase III clinical studies are undertaken and an application for marketing authorization is filed. The basic principles for the production and control of vaccines are published in the WHO Technical Report Series (7, 14–18). Specific WHO guidelines and recommendations for particular vaccines are also available and should be consulted where appropriate.

WHO Recommendations and Guidelines are intended to be scientific and advisory in nature and to provide guidance for national regulatory authorities and for vaccine manufacturers. These documents may be adopted by national health authorities as definitive national regulations or used as the basis of such regulations. They are also used as the basis for deciding the acceptability of vaccines for purchase by United Nations agencies such as the United Nations Children's Fund (UNICEF) for use in global immunization programmes. Regulatory requirements for vaccines and other biologicals are also produced by other bodies, such as the European Agency for the Evaluation of Medicinal Products (EMA) and the US Center for Biologics Evaluation and Research (CBER) (19); these documents can be found on the appropriate web sites (www.ema.eu.int and www.fda.gov/cber). In addition, pharmacopoeial requirements, such as those of the

European Pharmacopoeia, are also established for vaccines and are available at www.pheur.org.

For newly developed products, specific WHO, national or pharmacopoeial requirements may not be available and a national regulatory authority will need to agree on specifications with the manufacturer on a case-by-case basis during the evaluation of products for clinical trials and for licensing. For some of these novel products general guidance on production and control from WHO can be found in relevant documents, such as those describing DNA and peptide vaccines (14, 16), as well as recommendations on animal cell substrates used for production of biologicals (14).

In addition, information on how to assure the quality of biologicals in general and on procedures for approving manufacture and for setting up a national control laboratory, can be found in the relevant WHO guidelines (17, 18). For a vaccine intended to be marketed worldwide, the development of which also involves much international collaboration, it will be essential to ensure consistency of a regulatory approach for novel products such as vaccines for HIV prevention (19).

2.5 ***Batch release and independent laboratory evaluation***

The potential variability of methods for the production of biologicals has led to the establishment of national and international requirements to define procedures for assuring the quality of vaccines and for assessing consistency both among manufacturers and over long periods of time. Licensed vaccines are subject to independent batch release (review, testing and authorizing release of a batch of vaccine independent of the manufacturer) by a national regulatory authority or national control laboratory, before release on to the market. Independent evaluation entails at least an evaluation of a manufacturer's batch release data (protocol review), but in many instances it also includes independent laboratory testing in addition to that carried out by the manufacturer.

Batch or lot release tests are those tests chosen during full product characterization to demonstrate the purity, safety and potency of the product. Lot release testing provides one measure of assurance that a lot can be manufactured consistently. Validation and establishment of lot release tests and specifications is a process that continues throughout product development and should be finalized prior to licensure.

In some countries, samples of vaccine for clinical trials are required by the national regulatory authority, as a part of the approval process for clinical trials. Vaccine developers are encouraged to consult the

appropriate regulatory agency early on during the development of a vaccine.

2.6 **Standards and reference materials**

Standards and reference materials play a vital part in the licensing and quality control process, their role ranging from use in specific antigen recognition tests to assays of vaccine toxicity, immunogenicity and potency. The standardization of the methods used to evaluate vaccines, as well as those used to evaluate immune responses to vaccine antigens, is also vital so that results may be compared directly between laboratories both within and between countries, and between clinical trials.

WHO International Biological Standards and Reference Reagents are the primary standards in use worldwide. In addition, national regulatory authorities and manufacturers may establish secondary (regional, national), working standards for the purpose of testing vaccine quality on a lot-to-lot basis. Such standards should be calibrated against International Standards, when they exist. There is concern that different secondary standards may result in “drifting” from the International Standard. Production of secondary standards on a large scale (e.g. on a regional basis) reduces the number of secondary standards in use, and should improve accuracy of testing vaccine quality. For example, the European Department for the Quality of Medicines of the Council of Europe, has been active in establishing working standards for vaccines that are calibrated against the WHO International Standards, where appropriate. The complete list of WHO International Standards and Reference Reagents can be found on the WHO web site at: www.who.int/biologicals.

3 **Immunogenicity and other pharmacodynamic studies**

A pharmacodynamic study for a vaccine product is generally conducted to evaluate the immunogenicity. However, a pharmacodynamic study may also extend to include the pharmacology of an adjuvant.

Immunization studies in animal models should be conducted because they may provide valuable “proof of concept” information to support a clinical development plan. In addition, immunogenicity data derived from appropriate animal models are useful in establishing the immunological characteristics of the product and may guide selection of the doses, schedules and routes of administration to be evaluated in clinical trials. Nonclinical immunogenicity studies should assess the relevant immune response, e.g. humoral and/or cell-mediated

immune response, induced in the vaccinated animals. Depending on the immune response induced, such studies may include an evaluation of seroconversion rates, geometric mean antibody titres, or cell-mediated immunity in vaccinated animals. Nonclinical studies should, where possible, be designed to assess relevant immune responses, including functional immune response (e.g. neutralizing antibodies, opsonophagocytic activity, etc.) leading to protection. These studies may also be designed to address interference between antigens and/or live viruses. If a vaccine consists of more than one defined antigen (e.g. acellular pertussis vaccine consisting of 3–5 protein products) the response to each antigen should be evaluated. Where appropriate, challenge/protection studies with the corresponding infectious agent may be conducted to confirm the relevance of the animal models. A primary concern in interpreting the data obtained from such studies should be to determine how closely the animal model resembles the disease and immune response in humans. It should be recognized that animal models frequently fail to predict immunogenicity and efficacy in humans.

4 **Toxicity assessment**

The nonclinical safety assessment of vaccines needs to be viewed in the context of the evolving field of vaccine development. Thus, judgement based on the best science available should always form the basis for any decisions regarding the need for nonclinical safety studies, types of study and study designs. Similarly, scientific judgement should be applied to the interpretation of data from preclinical studies, regarding the risk–benefit ratio, animal model, dosing etc. For example, the observation of hypersensitivity reactions in an animal model may not necessarily preclude proceeding to clinical trials, but may indicate the necessity for careful monitoring of a particular clinical parameter.

Section 4.1 provides a general framework for designing a preclinical toxicity study for a vaccine. The parameters set out in this section are considered the minimum necessary for a safety assessment prior to the initiation of clinical trials in humans, in situations where preclinical safety studies are deemed necessary. As the design of any toxicity study is product-specific and based on indications, modifications to the framework outlined below may be necessary in response to particular product features, availability of animal models, methodologies, etc.

Section 4.2 provides additional considerations for performing special toxicity assessments that may be required on a case-by-case basis.

4.1 **Basic toxicity assessment**

4.1.1 *Study design*

The preclinical toxicity study should be adequate to identify and characterize potential toxic effects of a vaccine to allow investigators to conclude that it is reasonably safe to proceed to clinical investigation. The parameters to be considered in designing animal toxicology studies are the relevant animal species and strain, dosing schedule and method of vaccine administration, as well as timing of evaluation of end-points (e.g. sampling for clinical chemistry, antibody evaluation and necropsy). The route of administration should correspond to that intended for use in the clinical trials. When the vaccine is to be administered in human clinical trials using a particular device, the same device should be used in the animal study, where feasible (e.g. measles aerosol vaccine in the monkey model). Potential toxic effects of the product should be evaluated with regard to target organs, dose, route(s) of exposure, duration and frequency of exposure, and potential reversibility. The toxicity assessment of the vaccine formulation can be done either in dedicated-stand alone toxicity studies or in combination with studies of safety and activity that have toxicity end-points incorporated into the design. The study should also include an assessment of local tolerance.

4.1.2 *Animal species, sex, age and size of groups*

Data to be recorded on the animals used for toxicity testing should include information on the source, species and animal husbandry procedures (e.g. housing, feeding, handling and care of animals). In general, the use of outbred animals is recommended. The health of the animal will need to be evaluated in accordance with acceptable veterinary medical practice to ensure that animals are free of any condition that might interfere with the study. For instance, individual housing of laboratory animals may be required to minimize the risk of cross-infection.

Where possible, the safety profile of a product should be characterized in a species sensitive to the biological effects of the vaccine being studied. Ideally, the species chosen should be sensitive to the pathogenic organism or toxin. The animal species used should develop an immune response to the vaccine antigen. In general, one relevant animal species is sufficient for use in toxicity studies to support initiation of clinical trials. However, there may be situations in which two or more species may be necessary to characterize the product, for example where the mechanism of protection induced by the vaccine is not well understood (for example, intranasal influenza vaccine and intranasal measles vaccine).

In addition, when species-specific or strain-specific differences in the pharmacodynamics of the product are observed, it may be necessary to address the nonclinical safety of the product in more than one safety study and in more than one animal model.

The size of the treatment group depends on the animal model chosen. The number of animals used in studies using non-human primates would be expected to be less than that in studies that used rodents. For small animal models, e.g. rats and mice, it is recommended that approximately 10 males + 10 females per group be studied.

In general, the approximate age at the start of the study for rodents is 6–8 weeks, and for rabbits, 3–4 months.

4.1.3 *Dose, route of administration and control groups*

The toxicity study should be performed using a dose that maximizes exposure of the animal to the candidate vaccine and the immune response induced, for example, peak antibody response. In general, an evaluation of the dose–response is not required as part of the basic toxicity assessment and the lethal dose does not have to be determined. However, pilot dose–response studies may be conducted to determine which dose induces the highest antibody production in the animal model. If feasible, the highest dose (in absolute terms) to be used in the proposed clinical trial should be evaluated in the animal model. However, the dose is sometimes limited by the total volume that can be administered in a single injection, and guidelines on animal welfare should be followed. In such cases, the total volume may be administered at more than one site using the same route of administration. Alternatively, a dose that exceeds the human dose on a mg/kg basis and that induces an immune response in the animal model may be used. In such cases, the factor between human and animal dose should be justified.

The number of doses administered to the test animals should be equal to or more than the number of doses proposed in humans. To better simulate the proposed clinical usage, vaccine doses should be given at defined time intervals rather than as daily doses; the dosing interval used in the toxicity study may be shorter (e.g. an interval of 2–3 weeks) than the proposed interval in clinical trials in humans. The dosing interval in nonclinical trials may be based on the kinetics of the primary and secondary antibody responses observed in the animal model. A single-dose study may be performed in situations in which vaccine-induced antibodies are expected to neutralize a live viral vector, thus limiting the expression of the gene of interest (e.g. anti-adenovirus immune response), or when immune responses induced in

animals are expected to react with species-specific proteins present in the vaccine formulation (e.g. human recombinant cytokines used as adjuvants).

The route of administration should correspond to that intended for use in the human clinical trials. If toxic effects are observed in safety studies using a particular route of administration (e.g. intranasal), further toxicity studies using a different route of administration (e.g. intravenous) may be helpful in understanding the full spectrum of toxicity of the product.

The study design should include a negative control group(s) to evaluate a baseline level of treatment. If appropriate, active control groups (e.g. vaccine formulation without antigen) may also be included in the study. The study should include an additional treatment group of animals to be killed and evaluated as described below at later time-points after treatment, to investigate the reversibility of any adverse effects observed during the treatment period and to screen for possible delayed adverse effects.

4.1.4 *Parameters monitored*

Toxicity studies should address the potential of the product for causing local inflammatory reactions, and possible effects on the draining lymph nodes, systemic toxicity and on the immune system. A broad spectrum of information should be obtained from the toxicity studies. Parameters to be monitored should include daily clinical observations, weekly body weights and weekly food consumption. During the first week of administration frequent measurements of body weight and food consumption are recommended, if feasible, as these are sensitive parameters indicating “illness”. Interim analysis of haematology and serum chemistry should be considered approximately 1–3 days following the administration of the first and last dose and at the end of the recovery period. Haematology and serum chemistry analyses should include, at the minimum, an evaluation of relative and absolute differential white blood cell counts (lymphocytes, monocytes, granulocytes, abnormal cells) and albumin/globulin ratio, enzymes and electrolytes. In some cases, it may also be useful to evaluate coagulation parameters, urine samples and serum immunoglobulin classes. Data should be collected not only during treatment, but also following the recovery phase (e.g. 2 weeks or more following the last dose) to determine persistence, and look at exacerbation and/or reversibility of potential adverse effects.

At study termination, final body weights (after a period of fasting) should be measured. Terminal blood samples should be collected and

serum chemistry, haematology and immunological investigations should be done as described in the preceding paragraph. The immune response induced by the candidate vaccine should be assessed in order to confirm that the relevant animal model has been selected. A complete gross necropsy should be conducted and tissues collected and preserved, gross lesions should be examined and organ weights recorded (23). Histopathological examinations of tissues should be performed and special attention paid to the immune organs, i.e. lymph nodes (both local and distant from site of administration), thymus, spleen, bone marrow and Peyer's patches or bronchus-associated lymphoid tissue, as well as organs that may be expected to be affected as a result of the particular route of administration chosen. Histopathological examinations should always include pivotal organs (e.g. brain, kidneys, liver and reproductive organs) and the site of vaccine administration. The choice of tissues to be examined (ranging from a short list limited to immune and pivotal organs to a full list as provided in the Appendix) will depend on the vaccine in question, and the knowledge and experience obtained from previous nonclinical and clinical testing of the vaccine components. For example, full tissue examination will be required in the case of novel vaccines for which no prior nonclinical and clinical data are available. Therefore, the list of tissues to be tested should be defined on a case-by-case basis, following consultation with the relevant regulatory authority. Data should be reported in full listing the original collection of values, and summarized.

4.1.5 *Local tolerance*

The evaluation of local tolerance should be conducted either as a part of the repeated dose toxicity study or as a stand-alone study. Tolerance should be determined at those sites that come into contact with the vaccine antigen as a result of the method of administration, and also at those sites inadvertently exposed (e.g. eye exposure during administration by aerosol) to the vaccine. More details have been published elsewhere (24).

If abnormalities are observed in the basic toxicity study outlined in section 4.1., further studies may be necessary to evaluate the mechanism of the toxic effect.

4.2 **Additional toxicity assessments**

4.2.1 *Special immunological investigations*

In certain cases, the results from evaluations of immune response from nonclinical and clinical studies, or from data on natural disease, may indicate immunological aspects of toxicity, e.g. precipitation of

immune complexes, humoral or cell-mediated immune response against antigenic determinants of the host itself as a consequence of molecular mimicry or exacerbation of the disease (e.g. inactivated measles vaccine). In such cases, additional studies to investigate the mechanism of the effect observed might be necessary.

Great similarity of vaccine determinants and host molecules could cause autoimmune reactions induced by molecular mimicry (26). Therefore, any vaccine antigen whose characteristics might mimic those of a host antigen should be treated with caution, even though it is recognized that molecular mimicry does not necessarily predispose to autoimmunity.

Because considerable efforts may be required in selecting and developing relevant animal models to address the above issues, caution should be exercised and a strong rationale provided when developing vaccines for diseases associated with autoimmune pathology.

If data suggest that the pathogen against which the vaccine is directed may cause autoimmune pathology, studies may be needed to address this concern on a case-by-case basis, if an appropriate animal model exists.

It should be noted that observations of biological markers for autoimmune reactions are not necessarily linked to pathogenic consequences. For instance, the presence of autoimmune antibodies does not necessarily indicate the induction of autoimmune disease (25).

When hypersensitivity reactions induced by the antigen(s), adjuvants, excipients or preservatives are of concern, additional investigations may be warranted.

4.2.2 *Developmental toxicity studies*

Developmental toxicity studies are usually not necessary for vaccines indicated for immunization during childhood. However, if the target population for the vaccine includes pregnant women and women of childbearing potential, developmental toxicity studies should be considered, unless a scientific and clinically sound argument is put forward by the manufacturer to show that conducting such studies is unnecessary. For a preventive vaccine, reproductive toxicity assessments are generally restricted to prenatal and postnatal developmental studies, because the primary concern is any potential untoward effect on the developing embryo, fetus or newborn. The need to conduct fertility and post-weaning assessments should be considered on a case-by-case basis. The animal model chosen should develop

an immune response to the vaccine, which is usually determined by serum antibody measurements. In addition, it is important to evaluate maternal antibody transfer by measuring vaccine-induced antibody in cord or fetal blood to verify exposure of the embryo or fetus to maternal antibody. The route of administration should mimic the clinical route of administration. Ideally, the maximal human dose should be administered to the test animal. If it is not possible to administer the full human dose, e.g. limitations on the total volume that can be administered, or if local toxicity is observed that may result in maternal stress, a dose that exceeds the human dose on a mg/kg basis and is able to induce an immune response in the animal should be used.

To assess any potential adverse effects of the vaccine during the period of organogenesis, the gestating animal is usually exposed to the vaccine during the period from implantation until closure of the hard palate and end of gestation defined as stages C, D and E in the ICH S5a document (27). Because of the relatively short gestation period of most animal models used, pre-mating treatment is frequently required to ensure maximal exposure of the embryo or fetus to the vaccine-induced immune response. For a preventive vaccine, the number of doses administered depends on the time of onset and duration of the response. Booster immunizations may be necessary at certain times during the period of gestation to maintain a high level of antibody throughout the gestation period and to expose the developing embryo to the components of the vaccine formulation. End-points include, but are not limited to, viability, resorptions, abortions, fetal body weight and morphology. The reader is referred to other publications for guidance on end-points used to evaluate potential toxic effects of the product on development of the embryo or fetus (27). It is also recommended that a period of postnatal follow-up of pups from birth to weaning be incorporated in the study design to assess normality of growth, body weight gain, suckling activity and viability. Studies should therefore be designed so that test groups are divided into subgroups. Half of the animals should be delivered by Caesarean section and the other half allowed to deliver their pups without surgical intervention.

4.2.3 *Genotoxicity and carcinogenicity studies*

Genotoxicity studies are normally not needed for the final vaccine formulation. However, they may be required for particular vaccine components such as novel adjuvants and additives. If needed, the *in vitro* tests for mutations and chromosomal damage should be done prior to first human exposure. The full battery of tests for genotoxicity may be performed in parallel with clinical trials (28).

Carcinogenicity studies are not required for vaccine antigens. However, they may be required for particular vaccine components such as novel adjuvants and additives.

4.2.4 *Safety pharmacology*

The purpose of safety pharmacology is to investigate the effects of the candidate vaccine on vital functions. If data from nonclinical and/or human clinical studies suggest that the vaccine (e.g. one based on specific toxoids) may affect physiological functions (e.g. central nervous system, respiratory, cardiovascular and renal functions) other than those of the immune system, safety pharmacology studies should be incorporated into the toxicity assessment. Useful information on this topic can be found in the *Note for Guidance on safety pharmacology studies for human pharmaceuticals* (29).

4.2.6 *Pharmacokinetic studies*

Pharmacokinetic studies (e.g. for determining serum or tissue concentrations of vaccine components) are normally not needed. The need for specific studies should be considered on a case-by-case basis (e.g. when using novel adjuvants or alternative routes of administration) and may include local deposition studies that would assess the retention of the vaccine component at the site of injection and its further distribution (e.g. to the draining lymph nodes). Distribution studies should be considered in the case of new formulations, novel adjuvants or when alternative routes of administration are intended to be used (e.g. oral or intranasal).

5 **Special considerations**

5.1 ***Adjuvants***

Adjuvants may be included in vaccine formulations or co-administered with vaccines to enhance the immune responses to particular antigen(s), or to target a particular immune response. It is important that the adjuvants used comply with pharmacopoeial requirements where they exist, and that they do not cause unacceptable toxicity.

Adjuvant activity is a result of many factors and the immune response obtained with one particular antigen/adjuvant formulation cannot, as a rule, be extrapolated to another antigen. Individual antigens vary in their physical and biological properties and antigens may interact differently with an adjuvant. Adjuvants must be chosen according to the type of immune response desired and they must be formulated with the antigen in such a way that distribution of both is optimized to ensure availability to the relevant lymphatic tissues. The route of

administration of the vaccine is also an important factor influencing the efficacy and safety of an adjuvant.

The effect of the adjuvant should be demonstrated in preclinical immunogenicity studies. If no toxicological data exist for a new adjuvant, toxicity studies of the adjuvant alone should first be performed. In general, assessment of new or novel adjuvants should be undertaken as required for new chemical entity (30–32). These data may be obtained by the vaccine manufacturer or by the producer of the adjuvant. In addition to assessing the safety of the adjuvant by itself it is also important to assess whether the combination of antigen and adjuvant exerts a synergistic adverse effect in the animal model (33, 34). When species-specific proteins (e.g. cytokines) are used as novel adjuvants, the issue of species-specific response should be considered.

When evaluating the safety profile of the combination of adjuvant and vaccine, the formulation proposed for clinical use should be used.

Compatibility of the adjuvant(s) (e.g. lack of immune interference) with all antigenic components present in the vaccine should be evaluated.

If applicable, adsorption of all antigenic components present in the vaccine should be shown to be consistent on a lot-to-lot basis. Potential desorption of antigen during the shelf-life of the product should be performed as a part of stability studies, the results reported and specifications set, as this may affect not only immunogenicity, but also the toxicity profile of the product.

It should be noted that no adjuvant is licensed in its own right, but only as a component of a particular vaccine.

5.2 ***Additives (excipients and preservatives)***

Where a new additive is to be used, for which no toxicological data exist, toxicity studies of the additive alone should first be performed and the results documented according to the guidelines for new chemical entities (31). The compatibility of a new additive with all vaccine antigens should be documented together with the toxicological profile of the final vaccine formulation under consideration in animal models as outlined in section 4.

5.3 ***Vaccine formulation and delivery device***

The vaccine formulation (i.e. liquid form, capsules or powder), as well as the delivery device, may have an impact on the uptake of

the vaccine, its effectiveness and safety. Ideally, the delivery device and vaccine formulation tested in an animal safety study should be identical to those intended to be used clinically. However, animal models in which delivery devices intended for clinical use can be tested may not be available. In these instances, in order to develop an appropriate animal model, it may be necessary to conduct pilot studies to define and optimize the conditions for drug delivery in the animal model before it can be used to assess the preclinical safety of the product.

5.4 ***Alternative routes of administration***

When using a vaccine formulation administered by alternative routes (e.g. intranasal, oral, intradermal, rectal and intravaginal routes), it can be assumed that their potency, relevant immunogenicity, tolerability, toxicity, and long-term safety may differ from that of products delivered by the parenteral route. Thus, when different routes of administration are proposed, nonclinical safety studies may have to be conducted using vaccine formulation and/or adjuvant alone in a suitable animal model to address the specific safety concerns associated with vaccine administration by these routes. Particular issues relevant to vaccines administered using alternative routes that may need to be considered are discussed below.

5.4.1 *Animal models*

A special consideration for vaccines administered by alternative routes should be the anatomy and physiology of the site of vaccine administration of the particular animal model chosen and its accessibility for the administration of the vaccine. For example, for intranasally administered products, the species chosen should ideally be receptive to spray administration of the product. In general, rabbits and dogs are useful test models for use of spray devices; however, their olfactory bulbs are highly protected and special techniques would be required to ensure that the test product reached this organ. Although mice and rats are useful models, intranasal administration to these species presents technical difficulties. Intranasal administration to non-human primates may be preferable, if they are susceptible to the infectious agent in question.

Depending on the level of concern regarding a particular route of administration or when there are species-specific differences between the animal models in their sensitivity to the candidate vaccine, it may be necessary to address the preclinical safety of the product in more than one safety study and in more than one animal model.

5.4.2 *Dose*

As the optimal dose derived from studies using the parenteral route of administration may differ from the dose used for alternative route(s) of administration, dose-finding studies may need to be conducted for a particular route of administration. Also, consideration should be given to the total volume of the vaccine administered as it may affect the outcome of the safety study. For example, intranasal administration of more than 5 µl of test preparation per nostril to a mouse would result in the test preparation being swallowed, rather than being adsorbed by the nasal mucosa.

5.4.3 *End-points*

The toxicity end-points would include those described in section 4 and may include additional outcome measures that would depend on the route of administration and specific concerns associated with the particular route and target organ. For example, if there is concern about the potential passage of vaccine components to the brain following intranasal administration, immunohistology and “in situ” methods and/or neurological assays and examinations may be necessary. For vaccines administered by inhalation, outcome measures may include pulmonary function tests and data on histopathology of the lungs. Considerable efforts may be required to develop appropriate methods to address potential safety concerns associated with the use of new routes of administration.

5.4.4 *Immunogenicity assessment*

The development of appropriate assays for measuring mucosal immune responses is critical for vaccines that are expected to function as mucosal immunogens because serological assays alone may not reflect the relevant immune response for a mucosal vaccine. Thus, in addition to measuring serological responses, it may be necessary to evaluate T cell responses, antibody-secreting cells and cytokine production. In addition, assays may need to be developed to assess the induction of local and systemic responses at sites distant from administration of the vaccine antigen.

6 **Specific considerations for particular types of vaccines**

In addition to the testing strategies outlined in sections 3, 4 and 5, studies may be necessary to address specific safety concerns associated with particular product types using suitable in vitro and in vivo test methods. The specific testing requirements for live attenuated and combination vaccines are discussed below. Detailed information regarding the production and control of other types of vaccine is available in the WHO guidance documents for production and con-

trol (13), and should be consulted. For example, in the recently developed guidelines for DNA (16) and synthetic peptide vaccines (18, 35), as well as for particular vaccines such as Hib conjugated vaccine (26), the issues relevant for nonclinical testing are discussed and should be considered in the development of an appropriate design for the nonclinical study of the vaccine in question.

6.1 ***Live attenuated vaccines***

An assessment of the degree of attenuation, and the stability of the attenuated phenotype, are important considerations for the nonclinical testing programme of a live attenuated vaccine. Laboratory markers of attenuation are invaluable for this purpose. These markers should be capable of distinguishing the attenuated vaccine from fully virulent wild-type strains and, ideally, of detecting partial reversion to full virulence. To assess the stability of the attenuation phenotype, the vaccine may be passaged under production conditions beyond the maximum passage number to be used for production. Stability of attenuation may also be assessed by passage under conditions that are outside the conditions to be used for vaccine production. For example, higher or lower temperatures may exert selection pressure for reversion to virulence. The marker(s) of attenuation may subsequently be used to qualify new vaccine seed preparations and to monitor the effect of any significant changes in production conditions of the attenuated phenotype.

If the wild-type organism is neurotropic, or if passages through neural tissue have been used in the attenuation of a virus vaccine, then a test for neurovirulence should be performed at least at the level of the vaccine seed. A neurovirulence test is not necessarily required for all live attenuated vaccines. The specifications for an appropriate neurovirulence test depend on the organism under test and should be capable of distinguishing the attenuated vaccine from fully virulent wild-type strains and, ideally, of detecting partial reversion to full virulence. Specific reference preparations may be needed for this purpose. Neurovirulence tests in small animal models may be acceptable.

If the live attenuated vaccine is based on a genetically modified organism, then an environmental risk assessment may be required as part of the preclinical evaluation. An investigation into the possible shedding of vaccine organisms following administration contributes to the environmental risk assessment. For all live attenuated vaccines, information on the likelihood of exchange of genetic information with non-vaccine strains may be required and suitable nonclinical tests may be designed to provide data for this purpose.

6.2 **Combined vaccines**

New combinations produced either by formulation or at the time of reconstitution of antigens or serotypes should be studied for appropriate immunogenicity in an animal model, if available, before initiation of human clinical trials (36, 37). Combined antigens should be examined by appropriate physicochemical means to evaluate possible changes to antigen properties on combination, such as degree of adsorption to aluminium adjuvants, as well as stability of the combination.

The immune response to each of the antigens in the vaccine should be assessed, including the quality of response and any potential interference and incompatibilities between combined antigens. It is preferable to study a new combination in comparison with the individual antigens in animals to determine whether augmentation or diminution of response occurs.

The need to evaluate the safety of the new combination in an animal model should be considered on a case-by-case basis. Such evaluation is likely to be necessary if there is concern that combining antigens and/or adjuvants may lead to problems of toxicity (e.g. novel adjuvant).

Similar consideration for nonclinical testing will also apply to cases where a new candidate single-component vaccine is developed from an already licensed combined vaccine (e.g. monovalent oral polio vaccine versus trivalent oral polio vaccine).

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The final draft (WHO/BS/03.1969) was prepared by Dr E. Griffiths, Dr M. Gruber, Dr D. Masset, Dr F. Verdier, Dr D. Wood and Dr I. Knezevic, following a meeting held in Geneva, 9–10 June 2003, and taking into account comments made by the Expert Committee on Biological Standardization at its meeting in February 2003 as well as comments made by the reviewers of the document.

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Appendix
List of tissues to be collected in a repeated dose toxicity study

adrenal glands
aorta
bone (femur) and articulation
bone (sternum) with bone marrow
bone marrow smears¹
brain
bronchi (main-stem)
caecum
colon
duodenum
epididymides
eyes
heart
ileum
injection site(s) (a sample should be taken from the area of injection)
jejunum
kidneys and ureters
larynx
liver
lungs
lymph node (mandibular)
lymph node (mesenteric)
mammary gland
oesophagus
optic nerves

¹ Bone marrow smears should be prepared at the scheduled necropsy for all animals including any moribund animals killed during the study. The smears should be fixed in methanol and then stained by the May-Grunwald-Giemsa method.

ovaries and oviducts
pancreas
parathyroid glands
Peyer's patches
pituitary gland
prostate
rectum
salivary glands (mandibular, parotid, sublingual)
sciatic nerves
seminal vesicles
skeletal muscle
skin
spinal cord (cervical, thoracic, lumbar)
spleen
stomach
testes
thymus
thyroid glands
tongue
trachea
ureters
urinary bladder
uterus (horns + cervix)
vagina
all gross lesions

