Annex 2

Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines

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Guidelines published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

Introduction

These Guidelines are intended to provide guidance to NRAs and manufacturers on the nonclinical and initial clinical evaluation of vaccine adjuvants and adjuvanted vaccines by outlining international regulatory expectations in this area. The Guidelines should be read in conjunction with existing WHO guidelines on nonclinical (*1*) and clinical (*2*) evaluation of vaccines. There is substantial diversity among vaccine adjuvants and adjuvanted vaccines and their nonclinical and clinical testing programmes will depend upon product-specific features and their clinical indications. Therefore, the following text is written in the form of WHO Guidelines instead of Recommendations. Guidelines allow greater flexibility than Recommendations with respect to specific issues related to particular adjuvanted vaccines.

Over the past decades, strategies and approaches for the development and delivery of vaccine antigens have been expanded. Some of these antigens are weakly immunogenic and require the presence of adjuvants for the induction or enhancement of an adequate immune response. Vaccines with aluminium-based adjuvants have been used extensively in immunization programmes worldwide and a significant body of safety information has accumulated for them (*3*, *4*). As the knowledge of immunology and the mechanisms of vaccine adjuvant action have developed, the number of vaccines containing novel adjuvants being evaluated in clinical trials has increased. Vaccines containing adjuvants other than aluminium-containing compounds have been authorized for use in many countries (e.g. human papillomavirus and hepatitis B vaccines), and a number of vaccines with novel adjuvants are currently under development, including, but not limited to, vaccines against human immunodeficiency virus (HIV), malaria and tuberculosis, as well as new-generation vaccines against influenza and other diseases. However, the development and evaluation of adjuvanted vaccines present regulatory challenges. Vaccine manufacturers and regulators have questions about the type of information and extent of data that would be required to support proceeding to clinical trials with adjuvanted vaccines and to eventual authorization.

Existing WHO guidelines on nonclinical evaluation of vaccines (*1*) provide valuable general guidance; however, they provide limited information specifically related to new adjuvants and adjuvanted vaccines. Some of the issues addressed here are also discussed in national or regional guidance documents (*5*, *6*). Given the importance and the complexity of the issues, this updated and more extensive guidance on the nonclinical and preclinical testing of adjuvants and adjuvanted vaccines should allow manufacturers and regulators to proceed in an efficient manner on the critical path towards development and licensure of adjuvanted vaccines indicated for the control of diseases with an important global public health impact.

Background

Over the past decades, there have been a number of international workshops and meetings in which the issues covered by these WHO Guidelines have been discussed (*7–12*). To address the need for additional international guidance on nonclinical evaluation of adjuvanted vaccines, a consultation was organized by WHO on 7–8 September 2011 in Rockville, Maryland, United States, to initiate the process of developing new WHO guidance on the subject. The consultation was attended by experts from academia, NRAs, national control laboratories and industry involved in the research, manufacture and approval of adjuvanted vaccines from countries around the world. The purpose was to review the scientific information and available data and to discuss and identify the issues to be considered for the development of such international guidance. On 27–28 November 2012, WHO organized an informal consultation at its headquarters in Geneva, Switzerland attended by academics, researchers, vaccine manufacturers and regulators involved in the evaluation of adjuvanted vaccines, to review draft WHO Guidelines prepared by the drafting group and to seek consensus on key regulatory issues. The approaches to nonclinical and initial clinical evaluation of vaccine adjuvants and adjuvanted vaccines discussed in this document are a result of the efforts of this and other international working groups.

Scope

This document addresses regulatory considerations related to the nonclinical and initial clinical evaluation of adjuvanted vaccines. The goal of this document is to provide consistent and harmonized guidance on nonclinical testing approaches to support the use of candidate adjuvanted vaccines in all stages of clinical development and ultimately for marketing authorization of the product. However, each NRA may determine the regulatory requirements applicable for adjuvanted vaccines to be marketed and used in their country.

Vaccine adjuvants are substances or combinations of substances that are used in conjunction with a vaccine antigen to enhance (e.g. increase, accelerate, prolong and/or possibly target) or modulate to a different type (e.g. switch a Th1 immune response to a Th2 response, or a humoral response to a cytotoxic T-cell response) the specific immune response to the vaccine antigen in order to enhance the clinical effectiveness of the vaccine (see "Terminology" section below). For the purposes of this document, the term "adjuvant" includes formulations that contain one individual adjuvant as well as adjuvant combinations that contain multiple adjuvants. These WHO Guidelines specifically address vaccine adjuvants that are either separate substances that are mixed with vaccine antigens and administered at the same time and location as the vaccine antigen, or immunostimulatory

moieties that are engineered by recombinant DNA technology to be an inherent part of the antigen molecule (e.g. fusion proteins) or the immunogen (e.g. vectored vaccines). In this context, it should be noted that no vaccine adjuvant is authorized in its own right, but only as a component of a particular adjuvanted vaccine. This document does not deal with the carrier proteins that are covalently linked to polysaccharide antigens in conjugate vaccines. Also, the immune enhancing properties that are intrinsic to certain vaccine antigen preparations, such as the naturally occurring adjuvant activity of whole-cell pertussis vaccines, are not considered "adjuvants" within this document.

This document covers adjuvanted vaccines used in both prophylactic and therapeutic indications against infectious diseases. Nevertheless, some of the principles outlined below may be applicable to the nonclinical and initial clinical testing of adjuvanted therapeutic vaccines for other indications as well (e.g. cancer).

Nonclinical evaluation, within the context of this document, refers to all in vivo (in animal) and in vitro testing performed before and during the clinical development of adjuvanted vaccines and includes product characterization, proof-of-concept and immunogenicity studies, as well as safety testing in animals. Preclinical testing specifically refers to the nonclinical testing done prior to initiation of any human testing and is a prerequisite to movement of a candidate adjuvanted vaccine from the laboratory to the clinic. Thus, for the remainder of this document, the term "preclinical" will be used only when referring specifically to the nonclinical evaluation done prior to the first-in-human clinical trials.

Many regulatory agencies, in addition to defining an adjuvant based on its immune-enhancing biological activity, provide a regulatory and/or legal classification for the adjuvant component of a vaccine (e.g. excipient, active ingredient or constituent material). It is possible that depending on the particular definition used by the regulatory authority, additional testing may be required. These regulatory and legal issues are specific for each regulatory authority and are beyond the scope of this document.

General considerations

Adjuvants have been used for decades to enhance the immune response to vaccine antigens (*7*). Possible benefits of administering antigens in conjunction with adjuvants include the induction of long-term protection, better targeting of effector responses, induction of long-term memory, reduction of the antigen amount and/or the number of vaccine doses needed for a successful immunization and optimization of the immune response for populations with poor responsiveness. For certain complex diseases, stimulation of cell-mediated immune responses appears to be critical, and adjuvants can be employed to

optimize a desired immune response, such as the induction of cytotoxic or helper T lymphocyte responses. In addition, certain adjuvants can be used to promote antibody responses in a relevant immunoglobulin class or at mucosal surfaces.

Successful preclinical evaluation of adjuvanted vaccines, including physicochemical characterization, proof-of-concept testing in animals, and toxicity testing, is an important step towards their clinical development. In addition, studies in animals are valuable tools to help select a safe dose, schedule and route of administration, and to identify unexpected or potential adverse effects for specific monitoring in clinical trials. Safety concerns include potential inherent toxicities of the vaccine antigen and/or adjuvant, potential toxicities of any impurities and contaminants, and potential toxicities due to interactions of the components present in the final formulation. The regulatory considerations for adjuvanted vaccines are similar to those for vaccines in general, with additional issues being considered that are unique to novel adjuvants. For the purposes of these WHO Guidelines, a novel adjuvant is defined as an adjuvant that has not been included in a licensed vaccine.

Throughout this document, guidance is provided related to the evaluation of new adjuvants and adjuvanted vaccines, to include:

- unlicensed adjuvanted vaccines;
- antigens and adjuvants that have been included in licensed vaccines, but for which the production process has undergone significant changes;
- previously licensed products that have undergone major formulation changes (e.g. a change in adjuvant or addition or removal of one of the components);
- previously licensed products given by a new route of administration.

Where appropriate, considerations specific to the evaluation of novel adjuvants will be provided.

The established benefits and increased availability of adjuvants have stimulated an interest in transferring adjuvant production technology from one adjuvant or adjuvanted vaccine manufacturer to another. As stated above, adjuvants are not approved in their own right. In the context of vaccines against infectious diseases, adjuvants may only exist as components in licensed vaccines that consist of specific antigen/adjuvant combinations. Thus, each new adjuvanted vaccine is considered a new entity that will require appropriate physicochemical characterization and nonclinical and clinical evaluations. However, in cases of technology transfer, existing data from similar antigen and adjuvant components and/or adjuvanted vaccines held by the original manufacturer can provide important information to guide and potentially accelerate the nonclinical and clinical studies (e.g. data from adjuvant-alone study arms). The need for and

extent of nonclinical testing will depend on the adjuvanted vaccine under consideration; manufacturers are encouraged to consult with the NRA regarding the nonclinical testing needed.

Vaccine adjuvants have been divided broadly into two main types – those known as vaccine delivery systems, which enhance the delivery of the antigen to the local lymph node, and those known as immunostimulators, although this division has become less clear since some delivery systems are now known to have direct immune stimulatory effects in addition to their ability to enhance the delivery of the antigen to the local lymph node. Delivery systems include, but are not limited to, particles, carriers, emulsions and liposomes. Immunostimulators in general include substances that enhance the immune response to vaccine antigens by activating the innate immune system, which usually sets off a cascade of events including, but not limited to, increased antigen uptake into antigen-presenting cells, increased release of stimulatory molecules such as cytokines and increased localization of the antigen in the local lymph node. Immunostimulators may include cytokines or other substances that are generally described as "immune potentiators" because they exert direct effects on immune cells.

Adjuvants also can be classified according to their source (e.g. synthetic or microbial-derived), mechanism of action and physical or chemical properties. A list of the most commonly described adjuvant classes, with specific examples, is provided in Appendix 1. It should be noted that a given vaccine adjuvant may be a combination adjuvant (see "Terminology" section below) that consists of multiple types of adjuvants and thus can fall into more than one of the listed categories.

Terminology

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

Adjuvanted vaccine: the complete formulation that includes one or more antigens, an adjuvant(s), and any additives (which may include, for example, excipients or preservatives), the administration of which is intended to stimulate the immune system to result in an immune response that leads to the prevention or treatment of an infection or infectious disease.

First-in-human trial: for the purposes of this document, this refers to the first evaluation in human subjects. Most commonly, the first-in-human clinical trials are carried out in small numbers of healthy and immunocompetent adults to test the properties of a vaccine, its tolerability and, if appropriate, clinical laboratory and pharmacological parameters. These trials are considered phase I trials (*2*) and are primarily concerned with safety.

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 (*1*, *13*).

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 in the marketing authorization. In these Guidelines, GMP refers to the current GMP guidance published by WHO (*14*, *15*).

Immunogenicity: the capacity of a vaccine/adjuvanted vaccine to induce antibody-mediated immunity, cell-mediated immunity and/or immunological memory.

In vitro studies: refers to studies that are conducted in a laboratory environment using components (e.g. serum, cells or tissues) that were originally obtained from a living organism.

In vivo studies: refers to studies that are conducted with living organisms.

Nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines: nonclinical testing includes all in vivo and in vitro testing performed before and in parallel with the clinical development of adjuvanted vaccines. Nonclinical testing includes product characterization, proof-of-concept studies and animal in vivo/in vitro toxicity testing. The potential toxicity of an adjuvanted vaccine should be defined not only prior to initiation of human trials, but throughout clinical development, if appropriate (see also the definition of preclinical evaluation of vaccine adjuvants and adjuvanted vaccines).

Novel adjuvant: a novel adjuvant is an adjuvant that has not been contained in a licensed vaccine.

Potency: a measure of biological activity, using a suitably quantitative biological assay, based on an attribute of the product (e.g. adjuvanted vaccine) that is believed to be linked to the relevant biological properties. Other measures of potency (e.g. physicochemical analyses) may be appropriate based on the nature of the products (e.g. polysaccharides).

Preclinical evaluation of vaccine adjuvants and adjuvanted vaccines: preclinical testing refers specifically to the nonclinical testing (see definition of nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines) done prior to the first-in-human clinical trials. Preclinical evaluation is a prerequisite to the initiation of clinical trials.

Process intermediates: the antigen(s) and the adjuvant(s) used to produce the formulated adjuvanted vaccine.

Product characterization: a full battery of physical, chemical and biological tests conducted for a particular product (e.g. adjuvanted vaccine). These tests include, but are not limited to, in-process control testing, testing for adventitious agents, testing of process additives and process intermediates, and lot-release testing (*1*).

Proof-of-concept studies: proof-of-concept studies as discussed in this document include the in vivo and in vitro nonclinical testing conducted to evaluate the immune response to the adjuvanted vaccine, the enhancement of the immune response to the antigen by the adjuvant and/or the demonstration of the resulting protection against challenge with the infectious agent targeted by the adjuvanted vaccine. For therapeutic vaccines, proof-of-concept studies would include, when possible, studies to evaluate the capacity to control or ameliorate disease and/or clear infection.

Protocol or study/trial plan: a document that states the background, rationale and objectives of the nonclinical study or clinical trial, and describes its design, methodology and organization, including statistical considerations, and the conditions under which it is to be performed and managed (*1*).

Raw materials: ingredients used to produce process intermediates.

Route of administration: the means by which the candidate adjuvanted vaccine is introduced to the recipient. Routes of administration for adjuvanted vaccines may include, for example, the intramuscular, subcutaneous, transcutaneous (with or without scarification), intradermal, oral, intranasal, inhaled (aerosol), intravenous, intranodal, intravaginal or intrarectal routes.

Safety: the relative freedom from direct or indirect harmful effect to animals or persons by a product when appropriately administered, taking into consideration the character of the product in relation to the condition of the recipient at the time.

Vaccine adjuvants: substances or combinations of substances that are used in conjunction with a vaccine antigen to enhance (e.g. increase, accelerate, prolong and/or possibly target) or modulate to a different type (e.g. switch a Th1 immune response to a Th2 response or a humoral response to a cytotoxic T-cell response) the specific immune response to the vaccine antigen in order to enhance the clinical effectiveness of the vaccine. It may be any of the types of substances identified as examples of adjuvants in Appendix 1. The term "adjuvant" is used throughout the document to include adjuvants that exist as one individual substance as well as combination adjuvants that consist of multiple adjuvants and sometimes other additives.

Vaccine and adjuvanted vaccine: the complete formulation that includes an antigen (or an immunogen, e.g. a plasmid DNA vaccine) and any additives such as adjuvants, excipients or preservatives, the administration of which is intended to stimulate the immune system to result in an immune response to the vaccine antigen leading to the prevention or treatment of an infection or infectious disease. When the vaccine contains an adjuvant, it may be referred to as an adjuvanted vaccine.

Vaccine antigen: the active ingredient in a vaccine (or generated by a vaccine) against which a specific immune response is raised. The vaccine antigen may be a live, attenuated preparation of bacteria, viruses or parasites; inactivated

(killed) whole organisms; crude cellular fractions or purified antigens, including recombinant proteins (i.e. those derived from recombinant DNA expressed in a host cell); polysaccharides and conjugates formed by covalent linkage of polysaccharides to components such as mutated or inactivated proteins and/or toxoids, synthetic antigens, or heterologous proteins expressed by plasmid DNA or viral or bacterial vectors. It may also be a combination of the antigens or immunogens listed above.

Part A. Manufacturing and quality considerations for the nonclinical and clinical evaluation of vaccine adjuvants and adjuvanted vaccines

Adjuvanted vaccine manufacturers are encouraged to discuss with the NRA the extent of the manufacturing and quality-related information necessary to support the intended use of the antigen, the adjuvant and the adjuvanted vaccine. The extent of information necessary to evaluate and assure the consistent safety and effectiveness of adjuvanted vaccines will vary with the phase of nonclinical and clinical investigation. Similarly, the nature and extent of the manufacturing controls needed to achieve, and testing needed to demonstrate, appropriate adjuvanted vaccine quality differ not only among the various phases of product development (that is, research, pilot, investigational and commercial manufacture) but also among the various phases of clinical evaluation.

A.1 Production, characterization and quality assurance of lots to be used in nonclinical pharmacology studies

It is generally accepted that nonclinical pharmacology studies (e.g. the proof-of-concept and mechanism-of-action studies) may be done as non-GLP studies, and that they are often conducted with research or pilot-scale lots of antigen, adjuvant and/or adjuvanted vaccine formulations. Also, these studies are often dose-optimization studies in which the antigen and adjuvant components may be provided in two separate containers to allow for the mixing of different amounts of each component prior to administration, and the generation of data that support the proposed dose of antigen and adjuvant to be used in the investigational adjuvanted vaccine. While the level of characterization of the lots of antigen and adjuvant used in these exploratory studies may be less extensive than those to be used in the nonclinical toxicology and clinical studies, the same raw materials should be used, where possible, in their preparation, and the source and any testing of the raw materials – for example, purity and assessment of levels of metal ions (such as copper) in aluminium-containing compounds – should be documented. Ideally, the lots of antigen and adjuvant used to formulate the final

product should be manufactured by the same process as the lots to be tested in the nonclinical toxicology studies. The general quality of the adjuvanted vaccine components (that is, antigen and adjuvant intermediates) used in the nonclinical pharmacology studies should be adequately characterized preliminarily. As the relationship between physical and chemical characteristics of the adjuvanted vaccine and its components and the immunogenicity and efficacy of the adjuvanted vaccine is not completely understood in many cases, biological characterization (i.e. through the use of biological assays) should complement the physical and chemical characterization of the intermediates and the adjuvanted vaccine (see section A.2 and Table 2.1).

A.2 Production, characterization and quality assurance of lots to be used in nonclinical toxicology studies and first-in-human clinical trials

Ideally, the lots of the antigen, the adjuvant, and the adjuvanted vaccine used in the nonclinical toxicology studies should be the same lots as those proposed for use in the first-in-human trials; these lots should be manufactured in compliance with the GMPs that are appropriate for phase I clinical trial materials (*16*, *17*). Additionally, the quality and stability of the antigen, adjuvant and final adjuvanted vaccine formulation should be characterized adequately prior to, if not in parallel with, their use in a toxicology study (see section A.2.1 and Table 2.1).

If use of the same lots is not feasible, the lots used for the nonclinical toxicology studies should be comparable to those proposed for use in the first-in-human trials with respect to manufacturing process, physicochemical data, formulation and stability. Where there are significant differences in the manufacture of the antigen or the adjuvant (or in the formulation of the adjuvanted vaccine) to be used in the nonclinical toxicology studies and the first-in-human clinical trial, a detailed description of the differences should be provided. This information will allow the NRA to evaluate the potential impact of such changes on the safety of the adjuvanted vaccine and to determine whether or not the differences are sufficient to warrant the conduct of additional toxicology studies to support the safety of the proposed clinical use.

With respect to the control and testing of adjuvanted vaccine lots manufactured for use in first-in-human clinical trials, emphasis should generally be placed on elements that assure the safety of subjects. This usually includes identification and control of the raw materials used to manufacture the antigen and the adjuvant. For this reason, Certificates of Analysis, with test specifications and results indicated, should be provided for ingredients that are acquired from contract suppliers for use in manufacturing the adjuvanted vaccine. For some adjuvanted vaccines, additional considerations related to the manufacturing and testing of the vaccine adjuvant and its individual components may be needed

to provide assurance that the adjuvant is manufactured consistently and has a consistent composition. This may apply particularly when one or more of the components of the adjuvant is biological in nature, when the vaccine contains a complex adjuvant mixture, or when the antigens are adsorbed to mineral salts or gels. Therefore, it is important to use established quality control procedures that ensure the consistent manufacture of adjuvants and antigens to be used in the preparation of adjuvanted vaccines. The antigen and adjuvant, or formulated adjuvanted vaccine, used in the first-in-human trial should be manufactured under GMPs that are appropriate for phase I clinical trial materials (*16*, *17*). Compliance with GMPs will ensure that the lots of antigen, adjuvant and adjuvanted vaccine are consistently manufactured and controlled to the quality standards appropriate to their intended use. Compliance with all aspects of GMPs will be required at the later stages of clinical development (*14*, *15*) as discussed below (see section A.3 and Table 2.1).

The clinical lot(s) of adjuvanted vaccine, or separate lots of antigen and adjuvant if provided in separate final containers, should be demonstrated to be stable for the duration of the clinical trial. Additionally, if the adjuvant is provided in a separate container (e.g. vial or syringe) to be used to reconstitute or be added to the antigen prior to vaccine administration, a detailed description of the procedure for mixing the components should be provided. A clear statement of the appropriate time and conditions for storage of the individual components and the final adjuvanted vaccine should be provided. Also, the appearance of the adjuvanted vaccine after mixing should be described, and stability data to support the storage of the adjuvanted vaccine up to the time of administration should be provided.

A.2.1 Analytical testing of adjuvant, antigen and adjuvanted vaccine

A detailed description of the adjuvant, antigen and adjuvanted vaccine should be provided and include information regarding the characterization conducted to assure the quality (e.g. identity, purity, sterility) and quantity of the antigen and adjuvant as well as the potency of the adjuvanted vaccine. It should be demonstrated that the adjuvant does not adversely affect the potency of the antigen upon mixing. In addition, information on the methods of manufacture and testing for the intermediates and final product, together with their preliminary release specifications, should be provided. Although it is not necessary to have validated methods for testing the lots of antigen and adjuvant or adjuvanted vaccine to be used in nonclinical toxicology studies and first-in-human clinical trials, the scientific background should justify the choice of the testing methods and the selected preliminary specifications. It is recommended that the NRA be consulted when designing analytical protocols appropriate for establishing the identity and quantity of the antigen(s), adjuvant(s) and any additives. It is important to

assess attributes of each of the antigen and the adjuvant components that may be relevant for adjuvant activity and adjuvanted vaccine potency. Additionally, the properties of the antigen and the adjuvant that are most indicative of stability, both when stored individually and as a formulated final adjuvanted vaccine, should be identified.

Assays used for characterization of the adjuvant may or may not be related to its mode of action, but should be adequate to ensure consistency of adjuvant production and to evaluate adjuvant stability. These may include, for example, assays for appearance, particle size distribution, presence of aggregates and pH for the adjuvant, and the amount of aluminium and degree of antigen adsorption for a vaccine adsorbed to an aluminium-containing compound. Analytical methods to evaluate the antigen and the adjuvant in an adjuvanted vaccine should be developed and validated as adjuvanted vaccine product development and clinical evaluation proceed. If relevant, the methods to be developed for characterization purposes should include, where possible, methods to assess compatibility and/or physical interactions between the antigen and adjuvant (and between the components of the adjuvant, if a combination adjuvant is used). Validation of these methods should be completed if they are intended for quality control batch release during later-stage clinical development or commercial distribution.

A quality-control test evaluating the potency of the final adjuvanted vaccine should be developed as one of the assays to assess consistency of manufacture. Depending on the type of potency assessment conducted on the adjuvanted vaccine and the requirements of the NRA, the assessment may or may not reflect the contribution of the adjuvant to the potency of the adjuvanted vaccine. If it does not, it will be important to conduct assessments of the identity and content of the adjuvant in the final adjuvanted vaccine. Also, the purity and sterility of the final adjuvanted vaccine will need to be assessed to ensure its safety. If the adjuvant or adjuvanted vaccine is tested for endotoxin via the Limulus amoebocyte lysate (LAL) test method, evidence that the adjuvant or adjuvanted vaccine does not interfere with the LAL test (e.g. data from lipopolysaccharide spiking experiments with and without adjuvant) should be provided, as certain adjuvants, such as cationic liposomes, may interfere with the LAL test method. If interference is observed, alternative tests (e.g. pyrogen test or macrophage-activation test) should be investigated.

If the final adjuvanted vaccine consists of co-packaged antigen and adjuvant, where each is provided in a separate container to be mixed prior to administration, both the antigen and the adjuvant should be evaluated prior to mixing for relevant parameters, such as identification, purity and sterility. In addition, the potency of the antigen and the content of the adjuvant per dose should be assessed. Also, where feasible, evidence should be provided as mentioned previously to demonstrate that the adjuvant does not adversely affect the potency of the final adjuvanted vaccine. Thus, the potency of the extemporaneously mixed,

adjuvanted vaccine formulation should be demonstrated. For some adjuvanted vaccines (e.g. aluminium-adsorbed vaccines), it may not be possible, depending on the nature of the potency assay, to evaluate the potency of the final formulated vaccine by certain assays. In this case, the determination of the potency of the antigen alone prior to adsorption may be recommended as well as the development of an in vivo method for potency assessment of the final formulation.

Consultation with the NRA is recommended to discuss both the need for and design of the quality control test known as the innocuity, general safety, or abnormal toxicity test for the adjuvanted vaccine. Additionally, if a particular NRA requires such a test for a formulated adjuvanted vaccine, it should be clarified whether only the antigen or both the antigen and adjuvant are to be tested when provided in separate final containers. While some regulatory authorities and WHO no longer require this test to be performed on a routine basis once the consistency of production has been established, some have further questioned the relevance of this test (*18–20*). In some countries there is a legal requirement to conduct an innocuity test with the objective of assessing the potential introduction of extraneous impurities into the final adjuvanted vaccine; however, this is not considered a toxicity test. If the innocuity test is required, and the investigational adjuvant or adjuvanted vaccine does not pass the innocuity test when administered according to the prescribed protocol, which is typically volume based and administered by the intraperitoneal route, it will be necessary to define the appropriate dose and route of administration for the adjuvanted vaccine. The manufacturer of the vaccine will need to provide justification for a modification of the innocuity test in regulatory submissions. Such modifications should be discussed with the NRAs. In the countries where the innocuity test is still necessary, once test data from many lots have been accumulated, and consistency of production has been well established to the satisfaction of the NRA, it may be possible to request an exemption from conduct of the innocuity test as part of routine lot-release testing.

A.3 Information required for later-stage clinical trials

In general, in the course of adjuvanted vaccine product development, the analytical technology and methodology is developed in parallel with the clinical investigations. As the adjuvanted vaccine product development and clinical evaluation proceed, the quality control and quality assurance of the antigen and adjuvant should be refined. When clinical trials to collect safety and efficacy data to support licensure are initiated, the manufacturing processes should be demonstrated to be consistent and validated, and a detailed description with appropriate validation information should be provided for all analytical procedures (except for those that are from an official pharmacopeial compendium) (*14*, *15*). If a national or international standard is not yet available for a particular

antigen, adjuvant or adjuvanted vaccine, the manufacturer should establish its own primary reference material during later-stage clinical trials.

A minimum of three consecutive lots of each of the antigen and the adjuvant intermediates (or final containers if provided separately) and formulated adjuvanted vaccine should be manufactured and tested for purposes of demonstrating consistency of manufacture of the vaccine antigen, the adjuvant and the formulated adjuvanted vaccine. Any changes in the manufacture or formulation should be carefully assessed to determine if such changes directly or indirectly affect the quality or safety of the adjuvanted vaccine. When analytical data from tests conducted on the adjuvanted vaccine demonstrate that the antigen, adjuvant or adjuvanted vaccine manufactured before and after such changes is not comparable, additional qualification and/or bridging studies should be undertaken to support the safety of the materials proposed for continued clinical evaluation.

To ensure that appropriate stability data are collected during later stage clinical trials of the adjuvanted vaccine, a stability protocol to be used for the formal stability studies should be developed for the antigen, the adjuvant and the adjuvanted vaccine. Stability programmes should be designed to monitor the chemical, physical, biological and microbiological stability of the antigen, the adjuvant, and the adjuvanted vaccine throughout the clinical testing programme. The properties of each antigen and adjuvant that are most indicative of stability, both when stored individually and as a mixed final adjuvanted vaccine, should be identified as stability evaluations proceed (as mentioned in section A.2.1). If it is determined that degradation products accumulate from either the antigen or the adjuvant over the shelf-life of the adjuvanted vaccine, these should be evaluated during stability testing of the final product. It is recommended that the NRA be consulted to determine whether additional suitable nonclinical toxicological testing should be undertaken to confirm their safety. Additional guidance on stability testing of vaccines can be found in WHO Guidelines on stability evaluation of vaccines (*21*).

Part B. Rationale for the use of the adjuvant

Adjuvant activity is a result of multiple factors and an adjuvant-mediated enhancement of the immune response to one vaccine antigen, as a rule, cannot be extrapolated to the enhancement of the immune response to another antigen. Individual antigens vary in their physical, biological and immunogenic properties and antigens may have different needs for immunological help from an adjuvant (*5*). Manufacturers should justify the choice of the adjuvant based on the immune response desired, which may include effects on the magnitude, the breadth and/or the type of immune response to specific antigens and on the safety profile. In addition, adjuvants are also used in antigen dose-sparing strategies with the aim of

increasing the availability and supply of vaccines – for example, under emergency situations of an influenza pandemic (*22*) or as a strategy to decrease the cost of the vaccine (e.g. use of inactivated poliovirus vaccine for polio eradication) (*23*).

Many advances in the understanding of innate immunity have begun to provide insights into the immunological mechanisms of adjuvant action. Many of the immunostimulatory adjuvants are recognized by various members of the toll-like receptor (TLR) family, a subclass of pathogen-recognition receptors, while other adjuvants may target other families of pathogen-recognition receptors that could prove to be important in shaping the adaptive immune response. Furthermore, there are complex regulatory interactions between the many families of innate receptors and other signalling pathways. Within this framework, the activities exerted by adjuvants include, but are not limited to, the facilitation of: (a) mobilization of antigen-presenting and/or polymorphonuclear cells; (b) antigen uptake and presentation of the antigen(s) in the vaccine by antigen-presenting cells; (c) secretion of proteins by antigen-presenting cells; (d) recruitment, targeting and activation of antigen-specific cells; (e) modulation of activities that regulate the ensuing immune responses; and/or (f) protection of the antigen from degradation and elimination.

The scientific rationale supporting the benefit of adding the adjuvant and the choice of specific adjuvant(s) should be provided by the adjuvanted vaccine manufacturer. Before evaluating a particular adjuvant in combination with an antigen in a clinical trial, it is recommended that data from in vitro and/or in vivo studies be generated to support the rationale for including the specific adjuvant in the vaccine formulation and for selecting the dose range of adjuvant to be tested. In the ideal case, the mode of action of the selected adjuvant as well as the mechanism of the enhanced immune response would be well understood prior to the initiation of later-stage clinical development. When the mode of adjuvant action is not well defined, supplemental in vivo or in vitro data (as discussed in sections B.1 and B.2, respectively) may be provided in addition to the pivotal toxicity study to support the added benefit of the adjuvant to the immune response induced by the adjuvanted vaccine as well as the safety of the adjuvanted vaccine.

B.1 In vivo proof-of-concept studies

Data from proof-of-concept studies, including data from early studies conducted to evaluate optimal antigen/adjuvant formulations, can provide important information with regard to the characteristics of the adjuvanted vaccine. These data include evidence for the need for the adjuvant, the type and magnitude of the immune responses induced (i.e. innate immunity, or humoral and cellular immunity), and the functional capacity of the immune response to either protect against disease (i.e. prophylactic vaccine) or ameliorate an existing infectious disease (i.e. therapeutic vaccine) when a relevant nonclinical disease model

is available. These pilot or exploratory studies designed to identify and screen adjuvanted vaccine formulations may be non-GLP-compliant; however, they may identify unknown or potential adverse effects, and provide crucial information for the design of GLP-compliant toxicity studies. In addition, in vivo proof-of-concept studies may provide the scientific justification for manufacturing changes and for optimization of adjuvanted vaccine formulation, dose and route of administration during the clinical development of the adjuvanted vaccine product.

It is recommended that proof-of-concept studies to support the use of an adjuvant be carried out to evaluate vaccine formulations with and without the adjuvant. Depending on the specific antigen and/or adjuvant being considered, possible examples of these types of studies include:

- evaluation of humoral immune responses with regard to magnitude (e.g. mean titre or concentration), quality (e.g. affinity or avidity), and functional activity (e.g. neutralizing activity);
- evaluation of cellular immune responses including assessment of the induction of specific types of cellular responses (e.g. examining Th1 or Th2 cytokine profiles, or testing for the induction of cytotoxic T cells);
- evaluation of protective or therapeutic responses against the relevant pathogen using appropriate animal or in vitro disease models and/or evaluation of functional immune responses (e.g. neutralizing activity, serum bactericidal or opsonophagocytic antibody titres);
- evaluation of duration of (*24*) and extent of cross-protection provided by the induced immune response (*25*, *26*).

These studies will contribute to the elucidation of the adjuvant mode of action and may provide indication of the adjuvant-specific immune modulatory effects. In addition, these studies may assist in the interpretation of nonclinical safety studies and the identification of potential adverse effects to be monitored during clinical development. The development of in vitro model systems, particularly those using human cells, is recommended when possible, as they may provide additional relevant information to elucidate the mechanism of action of the adjuvant (see section B.2).

B.2 In vitro supporting studies

Functional in vitro bioassays may also provide helpful insight in understanding the mode of action of a particular adjuvant, and may provide valuable supplemental and complementary data to animal studies. This is particularly important when there are limitations to the animal models, such as species-specific differences (e.g. in TLRs). Antigen-presenting cells or other immune cells are widely used to assess and monitor the direct or indirect effects of adjuvants by measuring activation parameters (such as changes in the expression of cell

surface molecules and the pattern of cytokine secretion), and more recently such human cells have been used to develop in vitro assays that may be predictive of adjuvant safety in vivo (*27*). More complex tissue culture systems, containing a mixture of human immune cells mimicking lymphoid tissue, are being explored with the aim of evaluating human immune responses in vitro (*28*).

Part C. Considerations for selection of the animal species for nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines

Investigations of the properties that influence the safety and pharmacological activity of the adjuvant and the adjuvanted vaccine require the use of appropriate animal species. The animal species used for pharmacological and safety evaluations should be chosen carefully and justified. For ethical reasons, it is desirable to apply the 3Rs concept of "Replace Reduce Refine" to minimize the use of animals in research where scientifically appropriate (*29*). Both manufacturers and staff at the NRA or national control laboratory are encouraged to further develop in vitro assays and to evaluate their suitability for the control of vaccines (*30*).

C.1 Selection of animal species for nonclinical pharmacology studies

For the purpose of this document, the nonclinical pharmacological activity of an adjuvanted vaccine is defined as the ability of the adjuvanted vaccine to induce and/or modify an immune response in an animal species. Factors influencing the selection of a particular animal species include, but are not limited to, the vaccine antigen, the adjuvant chosen, the type of immunity (i.e. cell-mediated or humoral) to be induced and the route of administration. It is recommended that proof-of-concept studies be undertaken using an animal species in which: (a) an immune response to the vaccine antigen is developed; and (b) the immune response to the antigen is enhanced by the adjuvant through a mechanism similar to that expected in humans (e.g. TLRs known to be targeted by the adjuvant are present in the species, and enhanced humoral and/or cellular immunity is observed). However, it is acknowledged that species-specific differences in the immune responses induced in the animal species compared to the human are likely. Proof-of-concept studies most commonly are conducted in several animal species, including both naive and pre-exposed animals. In addition to evaluating the immune response induced by the vaccine antigen alone and in the presence of the adjuvant, the mechanism of action of the adjuvant in the absence of the vaccine antigen should also be evaluated.

If the adjuvanted vaccine is a therapeutic vaccine for an infectious disease indication, where feasible, disease animal models may need to be developed to

study the pharmacological activity of the adjuvanted vaccine and its effect on the disease. For preventive adjuvanted vaccines, the use, when available, of an animal species sensitive to the human pathogen may provide important insight into the mechanism of protection from the disease (e.g. the ferret model for human influenza).

Nonclinical pharmacology studies may be conducted under non-GLP compliant conditions. It is advisable to incorporate into the study design toxicological end-points to guide the design of GLP-compliant nonclinical safety studies. It is sufficient to conduct these studies in small animal species if it can be demonstrated that the animal species chosen is relevant and responsive to the vaccine antigen and the adjuvant when given by the intended route of administration. Nonhuman primates should be used only if no other relevant animal species is available.

C.2 Selection of animal species for nonclinical safety studies

When selecting the animal species for nonclinical safety studies, it is important to document the pharmacological activity of the vaccine in the presence and absence of adjuvant in that species. It is recommended that manufacturers conduct nonclinical safety studies in compliance with GLPs (see section D.2 and Table 2.1) and using an animal species in which an immune response to the vaccine antigen is developed and, ideally, the immune response to the antigen is enhanced by the adjuvant through a similar mechanism as expected in humans. It is not necessary, however, to conduct the nonclinical safety study in the same animal species used for proof-of-concept or nonclinical pharmacology studies (see sections B.1 and C.1). Nonhuman primates should be used only if no other relevant animal species is available. In situations where no animal species is available that is responsive to the adjuvanted vaccine, the choice of the animal species should be justified. In some circumstances, the use of in vitro model systems, particularly those using human cells, to evaluate the toxicity of the adjuvanted vaccine may provide additional supplementary information to assist in interpreting toxicity data (*27*).

It is highly recommended that the animal species chosen is one for which relevant and sufficient historical control data exist. Analysis and interpretation of data from the toxicity studies commonly includes a comparison with the inactive control (e.g. saline control) in the same study. However, historical control data from the same laboratory in which the study was conducted and for animals of comparable age and from the same species and/or strain may provide additional information. When historical control data are used, the data should be provided to the NRA.

The route of administration used in the toxicity study should correspond to that intended for use in the clinic. Also, when the adjuvanted vaccine is to be

administered in the clinic using a particular device, the same device should be used in the animal study, where feasible. For example, a small rodent species may not be an appropriate choice for nonclinical evaluation of a vaccine that is to be delivered intranasally because some of the inoculum could be delivered to the lungs. In this case, a larger animal or one with nasal surface area, anatomy and physiology similar to that of humans would be more appropriate.

Use of a single species is generally acceptable (see section D.2). This approach has commonly been accepted based primarily on pragmatic considerations – for example, the ability to predict the human immune response may be limited due to the species specificity of the response in animals to the antigen, the adjuvant or both.

C.3 Limitations of animal studies

The limitations of using animals to characterize the pharmacological and safety profile of an adjuvant or adjuvanted vaccine are acknowledged. The ability to predict the human immune response based on pharmacological studies in an animal may be limited due to the species specificity of the response to the antigen, the adjuvant, or both. Similarly, local and systemic adverse effects observed in a nonclinical safety study may not be directly translatable to the clinic. In addition, rare and/or late-onset adverse events that may occur in human subjects as a result of adjuvanted vaccine administration may not be observed in animal studies. Nevertheless, these studies offer the best currently available tools to evaluate the preclinical safety and pharmacology of adjuvanted vaccines.

D. Nonclinical safety assessment in animals

D.1 General remarks

Safety concerns for products such as vaccines include the potential inherent toxicities of the antigen and other vaccine components, as well as potential toxicities due to interactions of the components present in the final formulation. For adjuvanted vaccines, these concerns include the possibility that the immune-modulatory and/or inflammatory response induced may lead to undesired toxic side effects. Additionally, some adjuvants may elicit elevated levels of proinflammatory cytokines and other mediators of toxicity, irrespective of the immune response against the antigen.

Safety assessments in animal studies are valuable tools to help define an acceptable adjuvant/antigen ratio and a safe dose, as well as to identify unknown or potential adverse effects that should be taken into consideration for further product development or to be monitored in future clinical trials. The type of studies and the timing in relation to the clinical programme are presented in section D.2.

D.2 Toxicity studies of vaccine adjuvants and final adjuvanted vaccine formulations

The preclinical toxicity studies of the final adjuvanted vaccine formulation should be adequate to identify and characterize potential adverse effects of the vaccine in order to conclude that it is reasonably safe to proceed to first-in-human clinical investigation. As the mechanism of action of the adjuvant and/or adjuvanted vaccine formulation is often not fully understood, the toxicity studies should be designed to evaluate a broad spectrum of parameters due to the uncertainty of the in vivo effects and associated outcomes. Toxicity studies should be designed to mimic the intended route of administration in the clinic and to evaluate local reactogenicity (e.g. injection-site inflammation) and systemic toxicity (i.e. toxicity that occurs at sites distant from the site of initial administration). Pivotal toxicity studies should use the intended final formulation and dose of the adjuvanted vaccine (see section A.2) and should be conducted in compliance with GLPs.

When properly designed, conducted and interpreted, and when no major safety signals are revealed in the study results, one repeated-dose toxicity study in one relevant species should be sufficient. However, if there are significant manufacturing or formulation changes during product development, additional animal toxicity studies may be recommended to confirm that the safety profile of the product has not been changed. Also, throughout the clinical programme, additional animal toxicity studies (e.g. developmental and reproductive toxicity studies) may be necessary to investigate any adverse events observed in clinical trials or to support the use of the vaccine in a special population.

While comprehensive toxicity evaluations of the final adjuvanted vaccine formulation are considered essential, the advantages and limitations of toxicity studies with adjuvant alone have been discussed extensively in previous meetings and workshops (*7–11*). A comprehensive toxicity assessment of the adjuvant alone in animals (or of individual evaluations of its multiple components, if it is a combination adjuvant) may not be needed as a separate programme. However, to enable the interpretation of immunogenicity and safety studies of the adjuvanted vaccine, a study arm receiving adjuvant alone may be included in the repeated-dose toxicity studies (see section D.2.2) that are part of the comprehensive toxicity evaluations of the final adjuvanted vaccine formulation.

D.2.1 Safety pharmacology studies

The purpose of a safety pharmacology study is to investigate the effects of the candidate vaccine on vital functions. Although not usually required, safety pharmacology studies may be recommended by the NRA in some cases. For example, if data from nonclinical and/or human clinical studies suggest that the adjuvanted vaccine may affect physiological functions other than the immune

system (e.g. the central nervous system, respiratory or cardiovascular system, renal function or body temperature) then safety pharmacology studies should be incorporated into the safety assessment programme.

D.2.2 Repeated-dose toxicity studies

This section highlights important considerations regarding the study design for pivotal toxicity studies that should be conducted with the same vaccine formulation intended to be used in clinical trials (see section A.2). If more than one dose of an antigen or adjuvant is to be evaluated in the clinical study, the formulation containing the highest dose (i.e. the "worst case") should be included in the pivotal toxicity studies. Single-dose toxicity studies on the final formulated vaccine product, which are applicable to small-molecule chemical medicines, are usually not needed in accordance with *Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals: M3(R2)* (*31*). Acute effects of administering a vaccine can also be monitored in repeated-dose toxicity studies if they are adequately designed (e.g. an evaluation is conducted after the first administration). Alternatively, acute effects can be assessed in a single-dose design as part of a local tolerance study. For a study intended to support a first-in-human clinical trial, the number of animals studied per sex, group and time interval should be sufficient to allow meaningful scientific interpretation of the data generated. The size of the treatment group will depend on the animal species chosen; i.e. the number of animals included in studies using non-rodents (e.g. miniature pigs) would be expected to be fewer than the number included in studies using rodents. For mice and rats, it is recommended that at least 10 animals of each sex per group be used for the necropsy at the end of the treatment interval, and at least 5 animals of each sex per group be used for the necropsy at the end of the recovery period. For rabbits, it is recommended that at least five animals of each sex per group for each time interval be used. In general, the approximate age for rodents should be 6–8 weeks, and for rabbits, 3–4 months, at the start of the study.

D.2.2.1 Dose, dosing regimen and controls

Dose–response evaluation for the adjuvanted vaccine is generally not required as part of the basic toxicity assessment, given that, in most cases, dose–response assessment was explored in nonclinical pharmacology studies. For adjuvanted vaccines, the toxicity study should be performed using the highest anticipated human dose (in absolute terms) of the final adjuvanted vaccine to be used in the proposed clinical trial, where feasible. Ideally this dose provides optimal exposure of the animal to the candidate vaccine and the immune response induced. However, in the case of a novel adjuvant, it may be advisable to include additional (lower and higher) doses of the adjuvanted vaccine formulation or

adjuvant alone in order to identify a safe dose that could be used in a first-in-human clinical trial.

If the dose to be administered is limited by the total volume that can be administered in a single injection, guidelines for animal welfare should be followed (*32*). In such cases, the total volume may need to be administered at multiple sites using the same route of administration; however, it should be noted that the evaluation of local reactogenicity might be less reliable in such cases.

For adjuvanted vaccines intended to be given repeatedly, the number of doses administered to the animals in repeated-dose toxicity studies should equal or exceed the number of doses proposed in humans. However, in many cases, the studies are designed to include one dose more than planned for the clinical trial to allow for the possible inclusion of an additional dose in the clinical trial. To simulate the proposed clinical usage, vaccine doses should be given as episodic doses, but the dosing interval used in the toxicity study may be reduced (e.g. to 2 weeks or 3 weeks) compared with the proposed clinical dosing interval (which usually is greater than 2 weeks to 3 weeks). The nonclinical dosing interval should be based primarily on the kinetics of the primary and secondary antibody response observed in the animal study.

In general, the study design should include a negative control group that receives an inert placebo, such as saline, to evaluate a baseline level of treatment, and an adjuvant-alone arm to aid in the interpretation of safety data from the adjuvanted vaccine. Also, the treatment groups in the study should include a sufficient number of animals for evaluation (as described in section D.2.2.3) at later time points after treatment to evaluate the reversibility of adverse effects observed during the treatment period and to detect potentially delayed adverse effects.

D.2.2.2 Route of administration

The route of administration should correspond to that intended for use in the clinical trials. When the vaccine will be administered in human clinical trials using a particular device, the same device should be used in the animal study, where feasible.

D.2.2.3 End-points in toxicity studies

The following section discusses end-points that are especially relevant and important in the evaluation of adjuvanted vaccines in repeated-dose toxicity studies using the final vaccine formulation. In general, potential adverse effects of the adjuvanted vaccine should be evaluated in repeated-dose studies with regard to target organs (see Appendix 2), dose, route(s) of exposure, duration and frequency of exposure, and potential reversibility of observed toxic effects.

D.2.2.3.1 *Parameters for monitoring of systemic toxicity*

Toxicity studies, repeated-dose toxicity studies in particular, should address the potential for systemic toxicity including, but not limited to, the systemic effects on the immune system. A broad spectrum of information should be obtained from the toxicity study, and both in-life and postmortem data should be collected. This routinely includes careful monitoring of body weight and food consumption, body temperature, histopathology, clinical chemistry, haematology, coagulation parameters and acute phase reactants. In addition, the immune response should be evaluated in a group of treated animals to confirm that the anticipated immune response occurred during the toxicity study. A detailed description of the assay(s) used should be provided with the toxicity study results.

While the standard in-life parameters routinely assessed for general pharmaceuticals (e.g. overall health, body weight and food consumption) are appropriate, it is important to note that for adjuvanted vaccines more frequent (e.g. daily) measurements of body weight and food consumption are recommended, especially during the first week after the administration of each dose as these parameters are very sensitive in detecting systemic toxicity effects. After the first week, body weights may be collected less frequently (e.g. 2–3 times each week). Body temperature should also be evaluated prior to, and 3–8 h and 24 h after each dose. If there is an increase in temperature, additional measurements should be taken every 24 h until the values return to baseline. Interim analyses of haematology and serum chemistry should be considered within approximately 1–3 days following the first and last dose administration, and at the end of the recovery period; in addition, the collection of a predosing sample is recommended. Coagulation parameters should be included routinely; in some cases, evaluation of urine samples and serum immunoglobulin classes may be of value. Additionally, it is recommended that species-appropriate acute phase reactants (e.g. C reactive protein) be measured in the toxicity study prior to immunization, at time points following the administration of the adjuvant or adjuvanted vaccine that have been demonstrated to reflect peak elevations in the acute phase reactants being evaluated (commonly 24–48 h), and after a recovery phase of 7 days. When measuring acute phase reactants, the choice of the animal species may determine which proteins can be measured as these reactants vary among species (*33*). The data discussed above should be collected not only prior to and during the treatment phase, but also following the treatment-free (recovery) phase (i.e. 2 or more weeks following the last dose) to determine persistence, exacerbation and/or reversibility of potential adverse effects.

Postmortem data, including data from gross necropsy (with tissue collection and preservation, including gross lesions and organ weights), should be collected within 3 days following the last dose and following the above-mentioned recovery period (e.g. 2 or more weeks following the last dose) (*1*). At study termination, final body weights (following overnight fasting) should be

obtained. Terminal blood collection and analysis should include serum chemistry, haematology, and coagulation parameters as well as an immune-response evaluation. Histopathological examinations should always include pivotal organs (brain, lung, heart, kidneys, liver, reproductive organs), and the site of adjuvant or adjuvanted vaccine administration. Special attention should be paid to the immune organs – i.e. lymph nodes (draining and distant to the application site), thymus, spleen, bone marrow, and Peyer's patches or bronchus-associated lymphoid tissue – as well as organs that may be primarily affected due to the particular route of administration. The extent of the list of tissues to be examined (i.e. the full tissue list as provided in Appendix 2 versus the reduced list mentioned above, which is limited to the immune system and pivotal organs) will depend on the adjuvant or adjuvanted vaccine in question, as well as on the experience and knowledge obtained through previous nonclinical and clinical testing of the vaccine's components. Additionally, any known target organs of the adjuvant or adjuvanted vaccine should be evaluated. For novel adjuvants and adjuvanted vaccines containing a novel adjuvant, it is recommended that the full tissue list be evaluated.

D.2.2.3.2 *Parameters for monitoring of local reactogenicity*

Local toxicities should be determined at the site(s) of adjuvant or adjuvanted vaccine administration and any other sites that come into contact with the adjuvant or adjuvanted vaccine components as a result of the method of administration. Local toxicity studies of intramuscularly administered vaccines should preferably be conducted in animals with sufficient muscle mass to test the full human dose of the final vaccine formulation.

Injection site reaction after inoculation should be scored using a prospectively defined system (e.g. the modified Draize test) (*34*) along with an assessment of any vesiculation, ulceration, severe eschar formation and other manifestations of significant toxicity (e.g. limb impairment).

The site of administration and any other site that comes in contact with the adjuvant or adjuvanted vaccine (e.g. eye exposure during aerosol administration, or digestive tract after oral administration) should also be evaluated histopathologically. In addition, a description of cellular infiltrates based on routine histological staining, if present, should be reported as part of the postmortem evaluation, as well as any manifestation of tissue damage at the site of injection and surrounding anatomic structures (e.g. sciatic nerves, nasal cavities or olfactory bulb).

D.2.3 Developmental and reproductive toxicity

Because vaccination programmes may include women of childbearing potential, it is important to consider the need for developmental and reproductive toxicity studies. As is the case for general toxicity, the use of a novel adjuvant may require

adding an adjuvant-alone arm to the reproductive toxicity studies. However, the study design is also dependent on the intended clinical use of the vaccine. For example, vaccination may be given early in pregnancy to protect the mother at risk, or might be given later in pregnancy to induce passive immunization to protect the infant directly from birth.

In general, the administration of one or several additional doses during organogenesis (i.e. implantation to closure of the hard palate) is recommended in order to evaluate the potential, direct embryotoxic effects of the components of the vaccine formulation, and, depending on the animal model, to allow maternal antibody to transfer to the progeny during pregnancy or the lactation period. Depending on the adjuvant, there may be concern about an adjuvant-induced systemic inflammatory response, such as fever, which may adversely affect early pregnancy (e.g. implantation or placental growth) (*35*). In these cases, it is recommended to include in the study design an additional treatment group to evaluate the effect of adjuvant on early pregnancy parameters. Rather than dosing this treatment arm prior to mating, it is recommended to dose animals post-mating and prior to implantation (e.g. post-mating day 1). Considering the short gestational period of the animal species that are most frequently used, it may be necessary to administer priming doses to the animals several days or weeks prior to mating in order to elicit a peak antibody response during the period of organogenesis.

End-points in embryo-fetal/perinatal-postnatal toxicity studies include, but are not limited to, viability, abortions, number of resorptions, fetal body weight, morphology, preweaning development and growth, as well as survival incidence and developmental landmarks. For details on such studies, please see the United States Food and Drug Administration's *Guidance for industry: considerations for developmental toxicity studies for preventive and therapeutic vaccines for infectious disease indications* (*36*) and WHO guidelines on nonclinical evaluation of vaccines (*1*).

In most cases, the developmental and reproductive toxicity studies can be performed in parallel to the clinical trials. However, some NRAs require that women of childbearing potential be excluded from large-scale late-stage clinical trials that are conducted prior to the completion of developmental and reproductive toxicity studies; other NRAs require the use of appropriate birth control methods for women of childbearing potential that are included in clinical trials. Further considerations can be found in *Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals: M3(R2)* (*31*).

D.2.4 Biodistribution studies

Adjuvants are expected to exert their action locally in close connection to the antigen. However, biodistribution studies can be helpful in understanding the

distribution of the adjuvant following injection. The feasibility of and need for such biodistribution studies should be evaluated on a case-by-case basis.

D.2.5 Genotoxicity and carcinogenicity studies

Genotoxicity studies are normally not needed for the final vaccine formulation (*1*). However, a standard battery of genotoxicity studies is generally recommended for most novel adjuvants that are (or contain) new chemical entities (*31*, *37*). Based on previous experience, carcinogenicity studies are generally not needed for adjuvants or adjuvanted vaccines.

D.2.6 Toxicity studies of adjuvant alone

As noted in the introduction to section D.2, comprehensive toxicity assessment of the adjuvant alone in animals may be included as part of the study design with the adjuvanted vaccine. However, evaluation of the adjuvant alone can be important for novel adjuvants that have not been studied previously or will be used in multiple different vaccine formulations. In the case of a novel adjuvant or combination adjuvant, it may be advisable to include additional (lower and higher) doses of the adjuvant component(s) in order to identify a safe dose that could be used in a first-in-human clinical trial, as well as safety signals that should be monitored in the proposed clinical trial.

Although not usually required, safety pharmacology studies may be recommended in some cases to demonstrate that a novel adjuvant has no adverse effects on physiological functions (e.g. on the central nervous system, or the respiratory or cardiovascular system, renal function, and body temperature). If needed, such evaluations could also be included as a specific arm with the adjuvant alone in the repeated-dose toxicity study of the intended final vaccine formulation (*1*, *38*). It is expected that these studies would be conducted before initiating first-in-human clinical trials.

D.2.7 Summary of recommendations regarding timing of studies

In general, the guidance provided in this document regarding the timing of studies in relation to clinical trials is consistent with that of other guidance documents (*31*). A repeated-dose toxicology study (including safety pharmacology end-points, if needed) should be conducted before the first-in-human clinical trial. It may be important to conduct some studies with adjuvant alone (e.g. systemic toxicity and genotoxicity, when needed as discussed in sections D.2.5 and D.2.6) prior to initiation of clinical trials (*31*). Developmental toxicology studies should be performed prior to initiation of any clinical study to be conducted in pregnant women – i.e. for those vaccines specifically developed for use in pregnancy. For vaccines indicated for females of childbearing potential, subjects can be enrolled in clinical trials provided that appropriate precautions are taken to

avoid vaccination during pregnancy, such as pregnancy testing and use of birth control. For these products, developmental toxicity studies (section D.2.3) may be performed in parallel to the clinical study.

D.3 Additional considerations

Additional studies for safety assessment have been considered for the specific situation in which the target population for a vaccine containing a novel adjuvant includes very young subjects – such as neonates. At this time, however, there is insufficient knowledge about suitable animal models to evaluate whether neonates with an immature immune system would adequately respond to adjuvanted vaccines or whether the adjuvant could modify the neonatal immune system in an undesirable way. Modified immune responses to vaccination also have been observed in elderly populations; however, there also is insufficient knowledge about animal models to evaluate the response to adjuvants and adjuvanted vaccines in the ageing population. Further research to improve methods that can be used for the nonclinical evaluation of adjuvanted vaccines that are targeted for neonatal and elderly populations is encouraged.

Thus far, there is no compelling clinical evidence that adjuvants are causally related to the induction of autoimmune phenomena (or autoimmune disease) or hypersensitivity in humans (*4*). Although there has been interest in developing animal models that could be used to screen adjuvants and adjuvanted vaccines for induction of autoimmunity or hypersensitivity, such models do not currently exist. Therefore, no recommendations can be made at this time regarding specific nonclinical studies that should be conducted. These are complex and multifactorial conditions; further research is needed to identify additional biomarkers related to autoimmunity and hypersensitivity phenomena.

Part E. Considerations for first-in-human clinical trials

As with the nonclinical safety assessment considerations, the first-in-human trial considerations for new adjuvanted vaccines are similar to those for non-adjuvanted vaccines (*2*); however, some issues unique to the clinical evaluation of vaccines with novel adjuvants may need to be considered. The initial clinical trials of adjuvanted vaccines are usually intended to: (a) determine the subjects' tolerability to the range of doses of antigen and adjuvant, and the dosing regimen that may be needed for later immunogenicity and clinical end-point trials; and (b) to aid in the collection of information on the nature of the adverse reactions that can be expected. This section provides guidance on the points to consider when transitioning from nonclinical to clinical testing of adjuvanted vaccines as signals observed in nonclinical studies can aid in the design of the first-in-human clinical trials. This section is intended to supplement the information

provided in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (*2*).

Although there are limitations in the ability of animal and in vitro studies to predict safety in humans, all of the relevant nonclinical data, including the information on the pharmacologically active dose and the full toxicological profile of the adjuvanted vaccine, should be considered when designing the first-in-human trials. These data may aid in the selection of a safe starting dose, schedule, and route of administration, and in the identification of potential adverse effects for specific monitoring in the first-in-human clinical trial. A summary of such data from the nonclinical studies with the adjuvanted vaccine, and any available clinical data from similar or related adjuvanted vaccines, should be provided to support the acceptability of the proposed first-in-human clinical trial design. If, for example, dose-limiting toxicity was observed with the adjuvanted vaccine in the animal studies and the studies were repeated with lower doses to identify a dose that was without adverse effect in animals, it would be important to point that out and to summarize the specific adverse effects observed in the nonclinical studies.

Manufacturers should provide a rationale and scientific support for the use of an adjuvant in their vaccine. This could include information supporting the "added benefit" of the adjuvant derived from nonclinical studies (e.g. in vitro assays and/or proof-of-concept studies in animal models, including relevant challenge models when available) conducted prior to the initiation of clinical trials. In addition, it is recommended that the early clinical evaluations of an adjuvanted vaccine be designed to include the evaluation of both antigen-alone and adjuvanted vaccine arms to demonstrate the added benefit of the adjuvant; such data may include, for example, evidence of enhanced immune responses or antigen sparing.

If the safety of the adjuvanted vaccine was evaluated in appropriately designed toxicology studies that were conducted in line with the recommendations outlined above, and if there were no adverse effects observed in the toxicology studies conducted, the human dose tested in the toxicology studies may be acceptable as the starting dose in the first-in-human trials. However, such clinical trials are often designed as dose-escalating studies where the antigen and/or the adjuvant are given at escalating doses. With this in mind, given the limitations of the animal studies, it may be prudent to consider using a safety factor (a safety factor of 10 has been used historically) and to divide the human dose tested in the toxicology studies by the safety factor to find the recommended starting dose, and then escalate the dose from there. While it is anticipated that the adjuvant may have an antigen-sparing effect, the first-in-human trials should be designed to attempt to establish whether the adjuvant is needed and, if so, the minimum dose of adjuvant that is necessary to achieve adequate immunogenicity.

Although an inactive control (placebo) group may not be required in the first-in-human trial of an adjuvanted vaccine, the inclusion of a group receiving an inactive control, such as inert saline placebo, in early-phase clinical trials will enhance interpretation of the initial safety data through control for placebo effects and circulating community-acquired illnesses. It is recommended that the inclusion of an adjuvant-alone arm be discussed with the relevant NRA as some regulatory authorities recommend that such arms be avoided for ethical reasons; in those cases, an antigen-alone control arm may be preferred.

As with first-in-human trials of non-adjuvanted vaccines, those for adjuvanted vaccines are usually conducted in a limited number of healthy, adult volunteers (e.g. aged 18–50 years) with safety as the primary objective. The number of subjects enrolled in these first-in-human clinical trials typically ranges from 20 to 80 subjects; however, depending on the study design, the formulation of adjuvanted vaccine to be studied, and other relevant factors, a lower or higher number of subjects may be enrolled. To aid in the overall risk/benefit evaluation of the adjuvanted vaccine, the subject population should be clearly defined by inclusion and exclusion criteria, and the subjects should be closely monitored for safety. The clinical protocol should contain a safety monitoring plan with details of active post-vaccination monitoring, and predefined toxicity criteria for assessing the severity of clinical and laboratory parameters (*39*). In addition, the plan for increasing the dose of antigen and adjuvant, with predefined stepwise criteria for doing so, should be included in the clinical protocol. Also, it is recommended, especially when a novel adjuvant is used, that safety monitoring be extended through 12 months following the last vaccination (where the last follow-up may be accomplished by a telephone call). In this regard, it is recommended that serum specimens be banked where possible for potential future assessment in the event of a serious adverse event, a new-onset medical condition, or an adverse event of special interest that develops later in the course of the first-in-human clinical trial.

Any safety data based on experience with the same adjuvant formulated with other vaccine antigens, if available, may assist in developing the safety monitoring plan for the adjuvanted vaccine. However, since the mode of action in humans for the adjuvant in the specific adjuvanted vaccine to be evaluated in the first-in-human trial is usually unknown, and adjuvants may exhibit a range of properties that induce complex immune responses, it is recommended that subjects in first-in-human trials of adjuvanted vaccines be asked about specific adverse events. This may include, for example, inquiries on local reactions (e.g. pain, redness, swelling, granuloma formation, abscess, necrosis and regional lymphadenopathy), systemic reactions (e.g. fever, nausea, diarrhoea, and malaise), immune-mediated toxicity (e.g. cytokine release, immune suppression and autoimmune disease), and teratology. Examples of

adverse events of "special interest" may include neuroinflammatory disorders (e.g. optic neuritis and transverse myelitis), musculoskeletal and connective tissue diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus and Wegener granulomatosis), and gastrointestinal disorders (e.g. Crohn disease and ulcerative colitis). Additionally, targeted laboratory assessments (e.g. C reactive protein, fibrinogen, antinuclear antibody, antineutrophil cytoplasmic antibodies, and rheumatoid factor) may aid in the evaluation of adverse events and medical conditions.

Table 2.1

Points to consider for the manufacturing and quality information to be provided for pharmacology studies, toxicology studies[a] and first-in-human trials

Considerations	Comment on information needed, by type of study		
	Pharmacology	Toxicology[a]	First-in-human trials
Quality information regarding raw materials[b]	Information regarding purity and source of raw materials is important	Information regarding purity and source of raw materials is important	Information regarding purity and source of raw materials is important
Production of intermediates and adjuvanted vaccine	Production of intermediates and adjuvanted vaccine may be small scale	Production of intermediates and adjuvanted vaccine may be small scale; ideally, the lots used for the toxicology study should be the same as those that will be used in the first-in-human trials (or the lots should be comparable to the lots that will be used in the first-in-human trials in terms of the manufacturing process and the controls)	Production of intermediates and adjuvanted vaccine may be small scale, but control of manufacture is important; intermediates and adjuvanted vaccine should be manufactured in compliance with the appropriate good manufacturing practices

Table 2.1 *continued*

Considerations	Comment on information needed, by type of study		
	Pharmacology	Toxicology[a]	First-in-human trials
Presentation	Adjuvanted vaccine components (or antigen and adjuvant intermediates) often are provided in separate containers to be mixed prior to use	Adjuvanted vaccine may be provided as a premixed formulation or as two components (in separate containers) to be mixed prior to administration	Adjuvanted vaccine may be provided as a premixed formulation or as two components (in separate containers) to be mixed prior to administration
Characterization	Characterization of material may not be extensive; usually general quality information (e.g. composition, purity, potency[c, d]) is provided	Material should undergo considerable characterization to include, for example, information on purity, physicochemical characteristics and potency;[c, d] also, stability should be assessed	Material should undergo considerable characterization to include, for example, information on purity, physicochemical characteristics and potency;[c, d] also, stability should be assessed

[a] Toxicology studies should be compliant with GLPs (see "Terminology" section above).

[b] Ideally, the raw materials should be the same throughout all of the studies: pharmacology, toxicology and first-in-human trials.

[c] If a potency assay has been developed for the adjuvanted vaccine, such information should be provided. Alternatively, testing the antigen for potency, and the adjuvant for identity and content, is recommended.

[d] If the adjuvanted vaccine is provided premixed in one container, it should be tested for potency. However, in some cases, the potency assessment of the adjuvanted vaccine may require multiple types of tests (e.g. in the case of aluminium-adsorbed vaccines). In these cases, the determination of potency and amount of antigen present in the antigen intermediate preparation prior to adsorption (as well as the completeness of adsorption) may be recommended in addition to an in vivo method to assess the potency of the adjuvanted vaccine.

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References

1. WHO guidelines on nonclinical evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
2. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: *WHO Expert Committee on Biological Standardization*. *Fifty-second report*. Geneva, World Health Organization, 2004 (WHO Technical Report Series, No. 924), Annex 1.
3. Global Advisory Committee on Vaccine Safety, June 2012. *Weekly Epidemiological Record*, 2012, 87:281–287.
4. Ahmed SS et al. Assessing the safety of adjuvanted vaccines. *Science Translational Medicine*, 2011 3(93):93rv2 (doi: 10.1126/scitranslmed.3002302).
5. Committee for Medicinal Products for Human Use. *Guideline on adjuvants in vaccines for human use*. London, European Medicines Agency, 2005 (EMEA/CHMP/VEG/134716/2004) (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003809.pdf, accessed 26 June 2013).
6. *Guideline for nonclinical studies of preventive vaccines for infectious diseases: notification of yakushokushinsahatsu No. 0527-1 May 27*. Tokyo, Japan, Ministry of Health, Labour and Welfare, 2010 (Supplemental Information).
7. Global Advisory Committee on Vaccine Safety, 10–11 June 2004. *Weekly Epidemiological Record*, 2004, 79:269–272.
8. *Workshop on: non-clinical safety evaluation of preventive vaccines: recent advances and regulatory considerations*, vol. I. Arlington, VA, Center for Biologics Research and Review, United States Food and Drug Administration, 2002 (http://www.fda.gov/downloads/biologicsbloodvaccines/newsevents/workshopsmeetingsconferences/transcriptsminutes/ucm054459.pdf, accessed 26 June 2013).
9. van der Laan JW et al. Nonclinical testing of vaccines: report from a workshop. *Drug Information Journal*, 2009, 43:97–107.
10. *Workshop on adjuvants and adjuvanted preventive and therapeutic vaccines for infectious disease indications*. Arlington, VA, Center for Biologics Research and Review, United States Food and Drug Administration, 2008 (http://www.fda.gov/downloads/biologicsbloodvaccines/newsevents/workshopsmeetingsconferences/ucm095708.pdf, accessed 26 June 2013).
11. Mastelic B et al. Mode of action of adjuvants: implications for vaccine safety and design. *Biologicals*, 2010, 38:594–601.
12. Sun Y, Gruber M, Matsumoto M. Overview of global regulatory toxicology requirements for vaccines and adjuvants. *Journal of Pharmacological and Toxicological Methods*, 2012, 65:49–57.
13. *Handbook: good laboratory practice (GLP): quality practices for regulated non-clinical research and development*, 2nd ed. Geneva, UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, 2009 (http://www.who.int/tdr/publications/documents/glp-handbook.pdf, accessed 26 June 2013).

14. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992 (WHO Technical Report Series, No. 822), Annex 1.

15. Good manufacturing practices: main principles for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Forty-fifth report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 961), Annex 3.

16. Good manufacturing practices: supplementary guidelines for the manufacture of investigational pharmaceutical products for clinical trials in humans. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-fourth report*. Geneva, World Health Organization, 1996 (WHO Technical Report Series, No. 863), Annex 7.

17. *Guidance for industry: CGMP for phase 1 investigational drugs*. Rockville, MD, United States Food and Drug Administration, 2008 (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070273.pdf, accessed 26 June 2013).

18. Cussler K. A 4R concept for the safety testing of immunobiologicals. *Developments in Biological Standardization*, 1999, 101:121–126.

19. Gupta RK. Is the test for abnormal toxicity, general safety or innocuity necessary for vaccines? *Vaccine*, 1996, 14(17–18):1718.

20. Halder M. Three Rs potential in the development and quality control of immunobiologicals. *ALTEX*, 2001, 18(Suppl. 1):S13–S47.

21. Guidelines on stability evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-seventh report*. Geneva, World Health Organization, 2011 (WHO Technical Report Series, No. 962), Annex 3.

22. Friede M et al. WHO initiative to increase global and equitable access to influenza vaccine in the event of a pandemic: supporting developing country production capacity through technology transfer. *Vaccine*, 2011, 29(Suppl. 1):A2–A7.

23. Verdijk P, Rots NY, Bakker WA. Clinical development of a novel inactivated poliomyelitis vaccine based on attenuated Sabin poliovirus strains. *Expert Review of Vaccines*, 2011, 10:635–644.

24. Giannini SL et al. Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. *Vaccine*, 2006, 24:5937–5949.

25. De Vleeschauwer AR et al. Efficacy of an AS03A-adjuvanted split H5N1 influenza vaccine against an antigenically distinct low pathogenic H5N1 virus in pigs. *Vaccine*, 2012, 30:5557–5563.

26. Stephenson I et al. Cross-reactivity to highly pathogenic avian influenza H5N1 viruses after vaccination with nonadjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a potential priming strategy. *Journal of Infectious Diseases*, 2005, 191:1210–1215.

27. Zaitseva M et al. Use of human MonoMac6 cells for development of in vitro assay predictive of adjuvant safety in vivo. *Vaccine*, 2012, 30:4859–4865.

28. Ma Y et al. Assessing the immunopotency of Toll-like receptor agonists in an in vitro tissue-engineered immunological model. *Immunology*, 2010, 130:374–387.

29. Shin J et al. International regulatory requirements for vaccine safety and potency testing: a WHO perspective. *Procedia in Vaccinology*, 2011, 5:164–170.

30. Guidelines for independent lot release of vaccines by regulatory authorities. In: *WHO Expert Committee on Biological Standardization. Sixty-first report*. Geneva, World Health Organization, 2013 (WHO Technical Report Series, No. 978), Annex 2.

31. *Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals*. Tripartite Harmonised Guideline M3(R2). Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2009.

32. Diehl KH et al. A good practice guide to the administration of substances and removal of blood, including routes and volumes. *Journal of Applied Toxicology*, 2001, 21:15–23.

33. Kushner I, Mackiewicz A. The acute phase response: an overview. In: Mackiewicz A, Kushner I, Baumann H, eds. *Acute phase proteins: molecular biology, biochemistry, and clinical applications*. Boca Raton: CRC Press, 1993:3–19.

34. Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology and Experimental Therapeutics*, 1944, 82:377–390.

35. Herberts C et al. New adjuvanted vaccines in pregnancy: what is known about their safety? *Expert Review of Vaccines*, 2010, 9:1411–1422.

36. *Guidance for industry: considerations for developmental toxicity studies for preventive and therapeutic vaccines for infectious disease indications*. Center for Biologics Evaluation and Research, United States Food and Drug Administration, 2006 (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm092170.pdf, accessed 26 June 2013).

37. *Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use*. Tripartite Harmonised Guideline S2(R1). Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2011.

38. *Safety pharmacology studies for human pharmaceuticals*. Tripartite Harmonised Guideline S7A. Geneva, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2000.

39. *Guidance for industry: toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials*. Center for Biologics Evaluation and Research, United States Food and Drug Administration, 2007 (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm091977.pdf, accessed 26 June 2013).

Appendix 1

Examples of classes of adjuvants

The following main classes of adjuvants (see section on "Scope" and section 2 above) are currently used in licensed vaccines or are being investigated. The list is an updated version of the list of adjuvants developed by the European Medicines Agency, Committee for Medicinal Products for Human Use (*1*). For each category, representative examples are provided.

Classification of adjuvants

- **Mineral salts or gels** – for example, aluminium hydroxide, aluminium phosphate gels or calcium phosphate gels.
- **Oil-in-water and water-in-oil emulsions, amphiphilic molecules and surfactant-based formulations** – for example, Novartis' MF59 (microfluidized detergent-stabilized oil-in-water emulsion); QS-21 (purified saponin, which is derived from plants); GlaxoSmithKline's AS03 adjuvant (an oil-in-water emulsion plus α-tocopherol); and SEPPIC's Montanide ISA 51 and Montanide ISA 720.
- **Particulate adjuvants** – for example, liposomes; virosomes (unilamellar liposomal vehicles incorporating influenza haemagglutinin); DC Chol (a lipoidal immunostimulator able to self-organize into liposomes); immune-stimulating complexes known as ISCOMS (structured complexes of saponins and lipids) and CSL's Iscomatrix (the iscom without the incorporated antigen); and biopolymers such as Poly(lactide-co-glycolide) (PLGA).
- **Pathogen-associated molecular patterns (natural and synthetic)** – for example, low-toxicity versions of LPS, including monophosphoryl lipid A (MPL or MPLA) and RC-529 (a synthetic acylated monosaccharide); Detox adjuvant (an oil drop emulsion of MPL plus *Mycobacterium phlei* cell-wall skeleton); OM-174 (lipid A derivative); CpG motifs (synthetic oligodeoxynucleotides containing immunostimulatory CpG motifs); bacterial flagellin genetically fused with an antigen; bacterial toxins that have been genetically modified to provide nontoxic adjuvant effects such as modified heat-labile enterotoxin (LT) and cholera toxin (CT); and synthetic dsRNA such as Poly IC, Poly ICLC (also known as Hiltonol), and poly I:poly C12U (known as Ampligen).

- **Endogenous human immunostimulators** – for example, cytokines such as human granulocyte-macrophage colony-stimulating factor (hGM-CSF) or human interleukin-12 (hIL-12) that may be administered as proteins or as plasmid preparations (DNA sequences contained in DNA vaccine vectors that promote gene expression and are capable of inducing and/or promoting an immune response against an antigen in vaccine recipients).
- **Inert vehicles** – for example, gold particles.
- **Adjuvants derived from inulin** – for example, Vaxine's delta inulin (a plant-derived polysaccharide also known as Advax).
- **Combination adjuvants or adjuvant systems** consisting of combinations of vaccine-delivery systems and immunostimulatory agents that may result in more effective delivery of the immunostimulatory adjuvant as well as the antigen – for example, AS01 (liposomes, MPL and QS-21), AS02 (an oil-in-water emulsion plus MPL and QS-21), AS03 (an oil-in-water emulsion plus α-tocopherol), AS04 (MPL and aluminium hydroxide), AS15 (liposomes, MPL, QS-21 and a CpG oligodeoxynucleotide), glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE) (a synthetic acylated monosaccharide in a stable oil-in-water emulsion) and CAF01 (liposomes, a quaternary ammonium lipid and a synthetic analogue of a mycobacterial lipid).

Reference

1. Committee for Medicinal Products for Human Use. *Guideline on adjuvants in vaccines for human use*. London, European Medicines Agency, 2005 (EMEA/CHMP/VEG/134716/2004) (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003809.pdf, accessed 26 June 2013).

Appendix 2

Tissue samples to be collected for a repeated-dose toxicity study

This is a comprehensive list of the tissues that should be evaluated for local and systemic toxicity in repeated-dose toxicity studies; some additional tissues have been included to represent those specifically targeted by adjuvanted vaccines. This is an updated version of a list developed initially by WHO for vaccines (*1*) that was broadened and harmonized by the European Medicines Agency, Committee for Medicinal Products for Human Use (*2*) and the Society of Toxicologic Pathology (*3*).

Samples should be collected from the following tissues. The type of tissue to be collected depends upon the species used for testing.

adrenal glands

aorta (thoracic)

bone (femur) with articulation

bone (sternum) with bone marrow

bone marrow smears[1]

brain

bronchi (main stem)

caecum

colon

diaphragm

duodenum

epididymides

eyes

gall bladder

Harderian glands

heart

ileum

injection site(s) (a sample should be taken from the area of injection)

jejunum

kidneys

lachrymal glands (from the main body and subconjunctival part)

larynx

liver

lungs

lymph nodes that drain the injection site

lymph nodes that do not drain the injection site (e.g. mandibular or mesenteric)

mammary gland

nasal–oropharyngeal cavity (depending on the vaccine and adjuvant)

[1] Bone marrow smears should be prepared for all animals at the time of necropsy, including from any moribund animals killed during the study. The smears should be fixed in methanol and then stained using the May-Grunwald-Giemsa method.

nasal tissue (skull/nasal cavity)
oesophagus
optic nerves
ovaries
oviducts
pancreas
parathyroid glands
Peyer's patches
pituitary gland
prostate
rectum
salivary glands (mandibular, parotid and sublingual)
sciatic nerves
seminal vesicles
skeletal muscle
skin
spinal cord (cervical, thoracic and lumbar)
spleen
stomach
testes
thymus
thyroid glands
tissues with macroscopic observations (a sample should be taken from any and all tissues with macroscopic observations)
tongue
trachea
ureters
urinary bladder
uterus (from the body, horns and cervix)
vagina

References

1. WHO guidelines on nonclinical evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva, World Health Organization, 2005 (WHO Technical Report Series, No. 927), Annex 1.
2. Committee for Medicinal Products for Human Use. *Guideline on repeated dose toxicity*. London, European Medicines Agency, 2010 (EMEA/CHMP/SWP/1042/99 Rev. 1 Corr.) (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/03/WC500079536.pdf, accessed 26 June 2013).
3. Bregman CL et al. Recommended tissue list for histopathologic examination in repeat-dose toxicity and carcinogenicity studies: a proposal of the Society of Toxicologic Pathology (STP). *Toxicologic Pathology*, 2003, March–April, 31(2):252–253 (http://www.ncbi.nlm.nih.gov/pubmed/12696587, accessed 22 February 2014).