

Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines

Proposed guidelines

NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Publication of this draft is to provide information about the proposed WHO *Guidelines on the Nonclinical Evaluation of Vaccine Adjuvants and Adjuvanted Vaccines* to a broad audience and to improve transparency of the consultation process.

The text in its present form does not necessarily represent an agreed formulation of the Expert Committee. Written comments proposing modifications to this text MUST be received by 20 September 2013 in the Comment Form available separately and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Department of Essential Medicines and Health Products (EMP). Comments may also be submitted electronically to the Responsible Officer: **Dr TieQun Zhou** at email: zhout@who.int.

The outcome of the deliberations of the Expert Committee will be published in the WHO Technical Report Series. The final agreed formulation of the document will be edited to be in conformity with the "WHO style guide" (WHO/IMD/PUB/04.1).

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1 **Introduction**

2 This document provides guidance to National Regulatory Authorities (NRAs) and manufacturers
3 on the nonclinical and initial clinical evaluation of vaccine adjuvants and adjuvanted vaccines by
4 outlining the international regulatory expectations in this area. It should be read in conjunction
5 with the existing guidelines on nonclinical and clinical evaluation of vaccines published by the
6 WHO (1, 2). There is substantial diversity among vaccine adjuvants and adjuvanted vaccines
7 and their nonclinical and clinical testing programs will depend on product specific features and
8 their clinical indications. Therefore, the following text is written in the form of guidelines
9 instead of recommendations. “Guidelines” allow greater flexibility than “Recommendations”
10 with respect to specific issues related to particular adjuvanted vaccines.

11
12 Over the past decades, strategies and approaches for the development and delivery of vaccine
13 antigens have been expanded. Some of these antigens are weakly immunogenic and require the
14 presence of adjuvants for the induction or enhancement of an adequate immune response.
15 Vaccines with aluminum-based adjuvants have been used extensively in immunization programs
16 worldwide and a significant body of safety information has accumulated for them (3, 4). As the
17 knowledge of immunology and the mechanisms of vaccine adjuvant action have developed, the
18 number of vaccines containing novel adjuvants being evaluated in clinical trials has increased.
19 Vaccines containing adjuvants other than aluminum-containing compounds have been authorized
20 for use in several countries, and a number of vaccines with novel adjuvants are currently under
21 development, including, but not limited to, vaccines against human papillomavirus (HPV),
22 human immunodeficiency virus (HIV), malaria, and tuberculosis, as well as new generation
23 vaccines against influenza and other diseases. However, the development and evaluation of
24 adjuvanted vaccines presents regulatory challenges. Vaccine manufacturers and regulators have
25 questions about the type of information and extent of data that would be required to support
26 proceeding to clinical trials with adjuvanted vaccines and to eventual authorization. Existing
27 WHO guidelines on nonclinical evaluation of vaccines (1) provide valuable general guidance;
28 however, they provide limited information specifically related to new adjuvants and adjuvanted
29 vaccines. Additionally, some of the issues addressed here are also discussed in other
30 international guidance documents (5, 6). Given the importance and the complexity of the issues,

1 this up-dated and more extensive guidance on the nonclinical and preclinical testing of adjuvants
2 and adjuvanted vaccines should allow manufacturers and regulators to proceed in an efficient
3 manner on the critical path towards development and licensure of adjuvanted vaccines indicated
4 for the control of diseases with important global public health impact.

6 **Background**

7 Over the past decades, there have been a number of international workshops and meetings in
8 which the issues covered by these Guidelines have been discussed (7-12). To address the need
9 for additional international guidance on nonclinical evaluation of adjuvanted vaccines, a
10 consultation was organized by the World Health Organization (WHO) on 7-8 September 2011 in
11 Rockville, Maryland, USA, to initiate the process of developing a new WHO guideline on the
12 subject. The consultation was attended by experts from academia, National Regulatory
13 Authorities (NRAs), National Control Laboratories (NCLs) and industry involved in the
14 research, manufacture, and approval of adjuvanted vaccines from countries around the world.
15 The purpose was to review the scientific information and available data and to discuss and
16 identify the issues to be considered for the development of such international guidelines. On 27-
17 28 November 2012, the WHO organized an informal consultation in its headquarters in Geneva,
18 Switzerland, that was attended by academia, researchers, vaccine manufacturers and regulators
19 who are involved in the evaluation of adjuvanted vaccines, to review the draft guidelines
20 prepared by the drafting group and to seek consensus on key regulatory issues. The approaches
21 to nonclinical and initial clinical evaluation of vaccine adjuvants and adjuvanted vaccines
22 discussed in this document are a result of the efforts of this and other international working
23 groups.

25 **Scope**

26 This document addresses regulatory considerations related to the nonclinical and initial clinical
27 evaluation of adjuvanted vaccines. The goal of this document is to provide consistent and
28 harmonized guidance on nonclinical testing approaches to support the use of candidate
29 adjuvanted vaccines in all stages of clinical development and ultimately for marketing

1 authorization of the product. However, each NRA may determine the regulatory requirements
2 applicable for adjuvanted vaccines to be used and marketed in their country.

3
4 Vaccine adjuvants are substances or combination of substances that are used in conjunction with
5 a vaccine antigen to enhance (e.g., increase, accelerate, prolong and/or possibly target) the
6 specific immune response to the vaccine antigen and the clinical effectiveness of the vaccine.
7 For the purposes of this document, the term “adjuvant” includes formulations that contain one
8 individual adjuvant as well as adjuvant combinations that contain multiple adjuvants. These
9 Guidelines specifically address vaccine adjuvants that are either separate substances that are
10 mixed with the vaccine antigens and administered at the same time and location as the vaccine
11 antigens as well as immunostimulatory moieties that are engineered by recombinant DNA
12 technology to be an inherent part of the antigen molecule (e.g., in fusion proteins). This
13 document does not deal with the carrier proteins that are covalently linked to polysaccharide
14 antigens in conjugate vaccines. Also, the immune enhancing properties that are intrinsic to
15 certain vaccine antigen preparations, such as the naturally-occurring adjuvant activity of whole-
16 cell pertussis vaccines, are not considered “adjuvants” within this document.

17
18 This document covers adjuvanted vaccines used in both prophylactic and therapeutic indications
19 against infectious diseases. In this context, it should be noted that no vaccine adjuvant is
20 authorized in its own right, but only as a component of a particular adjuvanted vaccine.

21 Although these guidelines are primarily for preventive and therapeutic adjuvanted vaccines for
22 infectious disease indications, some of the principles outlined below may be applicable to the
23 nonclinical and initial clinical testing of adjuvanted therapeutic vaccines for other indications as
24 well (e.g., cancer).

25
26 Nonclinical evaluation, within the context of this document, refers to all *in vivo* (in animal) and
27 *in vitro* testing performed before and during the clinical development of adjuvanted vaccines and
28 includes product characterization, proof of concept and immunogenicity studies, as well as safety
29 testing in animals. Preclinical testing specifically refers to the nonclinical testing done prior to
30 initiation of any human testing and is a prerequisite to movement of a candidate adjuvanted
31 vaccine from the laboratory to the clinic. Thus, for the remainder of this document, the term,

1 preclinical, will be used only when referring specifically to the nonclinical evaluation done prior
2 to the first-in-human clinical trials.

3
4 Many regulatory agencies, in addition to defining an adjuvant based on its immune-enhancing
5 biological activity, provide a regulatory and/or legal classification for the adjuvant component
6 (e.g., excipient, active ingredient, or constituent material). It is possible that depending on the
7 particular definition used by the regulatory authority, additional testing may be required. These
8 regulatory and legal issues are specific for each regulatory authority and are beyond the scope of
9 this document.

10

11 **1. General considerations**

12 Adjuvants have been used for decades to enhance the immune response to vaccine antigens (7).
13 Possible benefits of administering antigens in conjunction with adjuvants include the induction
14 of long term protection, better targeting of effector responses, induction of long-term memory,
15 reduction of the antigen amount and/or the number of vaccine doses needed for a successful
16 immunization, and optimization for populations with poor responsiveness. For certain complex
17 diseases, stimulation of cell-mediated immune responses appears to be critical, and adjuvants can
18 be employed to optimize a desired immune response, such as the induction of cytotoxic or helper
19 T lymphocyte responses. In addition, certain adjuvants can be used to promote antibody
20 responses in a relevant immunoglobulin class or at mucosal surfaces.

21

22 Successful preclinical evaluation of adjuvanted vaccines, including physicochemical
23 characterization, proof of concept testing in animals, and toxicity testing, is an important step
24 towards their clinical development. In addition, studies in animals are valuable tools to help
25 select a safe dose, schedule, and route of administration, and to identify unexpected or potential
26 adverse effects for specific monitoring in clinical trials. Safety concerns include potential
27 inherent toxicities of the vaccine antigen and/or adjuvant, potential toxicities of any impurities
28 and contaminants, and potential toxicities due to interactions of the components present in the
29 final formulation. The regulatory considerations for adjuvanted vaccines are similar to those for
30 vaccines in general, with additional issues being considered that are unique to novel adjuvants.

1 For purposes of these Guidelines, a novel adjuvant is defined as an adjuvant that has not been
2 included in a licensed vaccine.

3
4 Throughout this document, guidance is provided related to the evaluation of new antigens,
5 adjuvants, and adjuvanted vaccines, to include:

- 6
- 7 • Unlicensed adjuvanted vaccines;
 - 8 • Antigens and adjuvants that have been included in licensed vaccines, but have undergone
9 significant changes in the production process;
 - 10 • Previously licensed products that have undergone major formulation changes (e.g., a
11 change in adjuvant or addition or removal of one of the components); and
 - 12 • Previously licensed products given by a new route of administration.

13
14 In some sections, specific considerations related to the evaluation of novel adjuvants will be
15 provided.

16
17 Established benefits and increased availability of adjuvants have stimulated an interest in
18 transferring adjuvant production technology from one adjuvant or adjuvanted vaccine
19 manufacturer to another. As stated above, adjuvants are not approved in their own right. In the
20 context of vaccines against infectious diseases, adjuvants may only exist as components in
21 licensed vaccines that consist of specific antigen/adjuvant combinations. Thus, each new
22 adjuvanted vaccine is considered a new entity that will require appropriate physicochemical
23 characterization and nonclinical and clinical evaluation. However, in cases of technology
24 transfer, existing data from similar antigen and adjuvant components and/or adjuvanted vaccines
25 held by the original manufacturer can provide important information to guide and potentially
26 accelerate the nonclinical and clinical studies (e.g., data from adjuvant alone study arms). The
27 need for and extent of nonclinical testing will depend on the adjuvanted vaccine under
28 consideration; manufacturers are encouraged to consult with the NRA.

29

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

13

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

20

21 **2. Definitions**

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

24

25 *Adjuvanted vaccine*

26 The complete formulation that includes one or more antigens, an adjuvant(s), and any additives,
27 (which may include, e.g., excipients or preservatives), the administration of which is intended to
28 stimulate the immune system to result in an immune response that leads to the prevention or
29 treatment of an infection or infectious disease.

30

1 *First-in-human trial*

2 For the purposes of this document, this refers to the first evaluation in human subjects. Most
3 commonly, the first-in-human clinical trials are carried out in small numbers (e.g., 20-80) of
4 healthy and immunocompetent adults to test the properties of a vaccine, its tolerability, and, if
5 appropriate, clinical laboratory and pharmacological parameters. These trials are primarily
6 concerned with safety.

7
8 *Good laboratory practice (GLP)*

9 A quality system concerned with the organizational process and the conditions under which
10 nonclinical health and environmental safety studies are planned, performed, monitored, recorded,
11 archived and reported. GLP principles may be considered as a set of criteria to be satisfied as a
12 basis for ensuring the quality, reliability and integrity of studies, the reporting of verifiable
13 conclusions and the traceability of data (1, 13).

14
15 *Good manufacturing practice (GMP)*

16 A part of the pharmaceutical quality assurance which ensures that products are consistently
17 produced and controlled according to the quality standards appropriate to their intended use and
18 as required in the marketing authorization. In these guidelines, GMP refers to the current GMP
19 guidelines published by WHO (14, 15).

20
21 *Immunogenicity*

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

24
25 *In vitro studies*

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

28
29 *In vivo studies*

30 *In vivo* studies refer to studies that are conducted with living organisms.

31

1 *Nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines*

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

8

9 *Novel adjuvant*

10 A novel adjuvant is an adjuvant that has not been contained in a licensed vaccine.

11

12 *Potency (1)*

13 The measure of biological activity, using a suitably quantitative biological assay, based on an
14 attribute of the product that is believed to be linked to the relevant biological properties.

15

16 *Preclinical evaluation of vaccine adjuvants and adjuvanted vaccines*

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

20

21 *Product characterization (1)*

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

25

26 *Process intermediates*

27 The antigen(s) and the adjuvant(s) used to produce the formulated adjuvanted vaccine.

28

29 *Proof of concept studies*

30 Proof of concept studies as discussed in this document include the *in vivo* and *in vitro* nonclinical
31 testing conducted to evaluate the immune response to the adjuvanted vaccine, the enhancement

1 of the immune response to the antigen by the adjuvant, and/or the demonstration of the resulting
2 protection against challenge with the infectious agent targeted by the adjuvanted vaccine. For
3 therapeutic vaccines, proof of concept studies would include, when possible, studies to evaluate
4 the capacity to control or ameliorate disease and/or clear infection.

5

6 *Protocol or study/trial plan (1)*

7 A document that states the background, rationale and objectives of the nonclinical study or
8 clinical trial, and describes its design, methodology and organization, including statistical
9 considerations, and the conditions under which it is to be performed and managed.

10

11 *Raw materials*

12 Ingredients used to produce process intermediates.

13

14 *Route of administration*

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

19

20 *Safety*

21 The relative freedom from harmful effect to animals or persons affected, directly or indirectly, by
22 a product when appropriately administered, taking into consideration the character of the product
23 in relation to the condition of the recipient at the time.

24

25 *Vaccine and adjuvanted vaccine*

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

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Vaccine adjuvants

Substances or combination of substances that are used in conjunction with a vaccine antigen to enhance (e.g., increase, accelerate, prolong and/or possibly target) the specific immune response to the vaccine antigen and 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 antigen

The active ingredient in a vaccine (or generated by a vaccine) against which a specific immune response is raised. It 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.

3. Manufacturing and quality considerations for 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 manufacturing controls needed to achieve and testing needed to demonstrate appropriate adjuvanted vaccine quality differ not only among various phases of product development (i.e., research, pilot, investigational and commercial manufacture), but also among the various phases of clinical evaluation.

1

2 **3.1 Production, characterization and quality assurance of lots to be used in** 3 **nonclinical pharmacology studies**

4 It is generally accepted that nonclinical pharmacology studies (e.g., the proof of concept and
5 mechanism of action studies) may be done as non-GLP studies and that they are often conducted
6 with research or pilot scale lots of antigen, adjuvant and/or adjuvanted vaccine formulations.

7 Also, these studies are often dose optimization studies in which the antigen and adjuvant
8 components may be provided in two separate containers to allow for the mixing of different
9 amounts of each component prior to administration and the generation of data that support the
10 proposed dose of antigen and adjuvant to be used in the investigational adjuvanted vaccine.

11 While the level of characterization of the lots of antigen and adjuvant used in these exploratory
12 studies may be less extensive than those to be used in the nonclinical toxicology and clinical
13 studies, the same raw materials should be used, where possible, in their preparation and the
14 source and any testing of the raw materials (e.g., purity and assessment of levels of metal ions
15 (e.g., copper) in aluminum-containing compounds) should be documented. Ideally, the lots of
16 antigen and adjuvant should be manufactured by the same methods as the lots to be tested in the
17 nonclinical toxicology studies. The general quality of the adjuvanted vaccine components (i.e.,
18 antigen and adjuvant intermediates) used in the nonclinical pharmacology studies should be
19 adequately characterized preliminarily. As the relationship between physical and chemical
20 characteristics of the adjuvanted vaccine and its components and the immunogenicity and
21 efficacy of the adjuvanted vaccine is not completely understood in many cases, biological
22 characterization (i.e., through the use of biological assays) should complement the physical and
23 chemical characterization of the intermediates and the adjuvanted vaccine. See Section 3.2
24 below and Table 1 for more information.

25

26 **3.2. Production, characterization and quality assurance of lots to be used in** 27 **nonclinical toxicology studies and first-in-human clinical trials**

28 The lots of the antigen, the adjuvant, and the adjuvanted vaccine used in the nonclinical
29 toxicology studies ideally should be the same lots as those proposed for use in the first-in-human
30 trials. Additionally, the quality and stability of the antigen, adjuvant and final adjuvanted

1 vaccine formulation should be characterized adequately prior to, if not in parallel with, their use
2 in a toxicology study. See Table 1 and Section 3.2.1 below for more information.

3
4 If use of the same lots is not feasible, the lots used for the nonclinical toxicology studies should
5 be comparable to those proposed for use in the first-in-human trials with respect to
6 manufacturing process, physicochemical data, formulation and stability. Where possible, the lots
7 used for the nonclinical toxicology studies should be manufactured under good manufacturing
8 practices (GMPs) that are appropriate for Phase 1 clinical trial material (16, 17). Where there are
9 significant differences in the manufacture of the antigen or the adjuvant (or in the formulation of
10 the adjuvanted vaccine) to be used in the nonclinical toxicology studies and the first-in-human
11 clinical trial, a detailed description of the differences and additional safety studies conducted to
12 compare the safety profiles should be provided. This information is provided to allow the NRA
13 to evaluate the potential impact of such changes on the safety of the adjuvanted vaccine and to
14 determine whether or not the differences are sufficient to warrant the conduct of additional
15 toxicology studies to support the safety of the proposed clinical use.

16
17 With respect to the control and testing of adjuvanted vaccine lots manufactured for use in first-
18 in-human clinical trials, emphasis should generally be placed on elements that assure the safety
19 of subjects. This usually includes identification and control of the raw materials used to
20 manufacture the antigen and the adjuvant. For this reason, Certificates of Analysis with test
21 specifications and results indicated should be provided for all ingredients that are acquired from
22 contract suppliers for use in manufacturing the adjuvanted vaccine. For some adjuvanted
23 vaccines, additional considerations related to manufacturing and testing of the vaccine adjuvant
24 and its individual components may be needed to provide assurance that the adjuvant is
25 manufactured consistently and has a consistent composition. This may apply particularly when
26 one or more of the components of the adjuvant is biological in nature, when the vaccine contains
27 a complex adjuvant mixture, or when the antigens are adsorbed to mineral salts or gels.
28 Therefore, it is important to use established quality control procedures that ensure the consistent
29 manufacture of adjuvants and antigens to be used in the preparation of adjuvanted vaccines. The
30 antigen and adjuvant or formulated adjuvanted vaccine used in the first-in-human trial should be
31 manufactured under GMPs that are appropriate for Phase 1 clinical trial material (16, 17).

1 Compliance with GMP will ensure that lots of antigen, adjuvant and adjuvanted vaccine are
2 consistently manufactured and controlled to the quality standards appropriate to their intended
3 use. Full GMP will be required at the later stages of clinical development (14, 15) as discussed
4 below (Section 3.3). See Table 1 for more information.

5
6 The clinical lot(s) of adjuvanted vaccine, or separate lots of antigen and adjuvant if provided in
7 separate final containers, should be demonstrated to be stable for the duration of the clinical trial.
8 Additionally, if the adjuvant is provided in a separate container (e.g., vial or syringe) to be used
9 to reconstitute or be added to the antigen prior to vaccine administration, a detailed description of
10 the procedure for mixing the components should be provided. A clear statement of the
11 appropriate time and conditions for storage of the individual components and the final
12 adjuvanted vaccine should be provided. Also, the appearance of the adjuvanted vaccine after
13 mixing should be described, and stability data to support the storage of the adjuvanted vaccine up
14 to the time of administration should be provided.

15 16 ***3.2.1 Analytical testing of adjuvant and adjuvanted vaccine***

17 A detailed description of the adjuvant and adjuvanted vaccine should be provided and include
18 information regarding the characterization conducted to assure the quality (e.g., identity, purity,
19 sterility) and quantity of the antigen and adjuvant as well as the potency of the adjuvanted
20 vaccine. It should be demonstrated that the adjuvant does not adversely affect the potency of the
21 antigen upon mixing. In addition, information on the methods of manufacture and testing for the
22 intermediates and final product, together with their preliminary release specifications, should be
23 provided. Although it is not necessary to have validated methods for testing lots of antigen and
24 adjuvant or adjuvanted vaccine to be used in nonclinical toxicology studies and first-in-human
25 clinical trials, the scientific background should justify the choice of the testing methods and the
26 selected preliminary specifications. The NRA should be consulted to define analytical protocols
27 appropriate for the identification and quantification of the content of the antigen(s), adjuvant(s)
28 and any additives. It is important to assess attributes of each of the antigen and the adjuvant that
29 may be relevant for adjuvant activity and adjuvanted vaccine potency. Additionally, the

1 properties of the antigen and the adjuvant that are most indicative of stability, both when stored
2 individually and as a formulated final adjuvanted vaccine, should be identified.

3
4 Assays used for characterization of the adjuvant may or may not be related to its mode of action,
5 but should be adequate to ensure consistency of adjuvant production and to evaluate adjuvant
6 stability. These assays may include, for example, appearance, particle size, presence of
7 aggregates, and pH for the adjuvant, and the amount of aluminum and degree of antigen
8 adsorption for a vaccine adsorbed to an aluminum-containing compound. Analytical methods to
9 evaluate the antigen and the adjuvant in an adjuvanted vaccine should be developed and
10 validated as adjuvanted vaccine product development and clinical evaluation proceeds. If
11 relevant, the methods to be developed for characterization purposes should include, where
12 possible, methods to assess compatibility and/or physical interactions between the antigen and
13 adjuvant (and between the components of the adjuvant, if a combination adjuvant is used).
14 Validation of these methods should be completed if they are intended for quality control batch
15 release during later stage clinical development or commercial distribution.

16
17 A quality control test evaluating the potency of the final adjuvanted vaccine should be developed
18 as one of the assays to assess consistency of manufacture. Depending on the type of potency
19 assessment conducted on the adjuvanted vaccine and the requirements of the NRA, the
20 assessment may or may not reflect the contribution of the adjuvant to the potency of the
21 adjuvanted vaccine. If it does not, it will be important to conduct assessments of the identity and
22 content of the adjuvant in the final adjuvanted vaccine. Also, the purity and sterility of the final
23 adjuvanted vaccine will need to be assessed to ensure its safety. If the adjuvant or adjuvanted
24 vaccine is tested for endotoxin via the Limulus Amoebocyte Lysate (LAL) test method, evidence
25 that the adjuvant or adjuvanted vaccine does not interfere with the LAL test (e.g., data from
26 lipopolysaccharide spiking experiments with and without adjuvant) should be provided, as
27 certain adjuvants, such as cationic liposomes, may interfere with the LAL test method.

28
29 If the final adjuvanted vaccine consists of co-packaged antigen and adjuvant where each is
30 provided in a separate container to be mixed prior to administration, both the antigen and the
31 adjuvant should be evaluated prior to mixing for identification, purity and sterility. In addition,

1 the potency of the antigen and the content of the adjuvant should be assessed. Also, where
2 feasible, evidence should be provided as mentioned previously to demonstrate that the adjuvant
3 does not adversely affect the potency of the final adjuvanted vaccine. Thus, the potency of the
4 extemporaneously mixed, adjuvanted vaccine formulation should be demonstrated. For some
5 adjuvanted vaccines (e.g., aluminum-adsorbed vaccines), it may not be possible, depending on
6 the nature of the potency assay, to evaluate the potency of the final formulated vaccine by certain
7 assays. In this case, the determination of potency of the antigen alone prior to adsorption may be
8 recommended as well as the development of an *in vivo* method for potency assessment of the
9 final formulation.

10

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

30

1 **3.3 Information required for later stage clinical trials**

2 In general, in the course of adjuvanted vaccine product development, the analytical technology
3 and methodology is developed in parallel with the clinical investigations. As the adjuvanted
4 vaccine product development and clinical evaluation proceeds, the quality control and quality
5 assurance of the antigen and adjuvant should be refined. When clinical trials to collect safety
6 and efficacy data to support licensure are initiated, the manufacturing processes should be
7 demonstrated to be consistent and validated and a detailed description with appropriate
8 validation information should be provided for all analytical procedures (except for those that are
9 from an official pharmacopeial compendium) (14, 15). If a national or international standard is
10 not yet available for a particular antigen, adjuvant, or adjuvanted vaccine, the manufacturer
11 should establish its own primary reference material during later stage clinical trials.

12
13 A minimum of three consecutive lots of each of the antigen and the adjuvant intermediates (or
14 final containers if provided separately) and formulated adjuvanted vaccine should be
15 manufactured and tested for purposes of demonstrating consistency of manufacture of each
16 component and the formulated adjuvanted vaccine. Any changes in the manufacture or
17 formulation should be carefully assessed to determine if such changes directly or indirectly affect
18 the quality or safety of the adjuvanted vaccine. When analytical data from tests conducted on the
19 adjuvanted vaccine demonstrate that the antigen, adjuvant or adjuvanted vaccine manufactured
20 before and after such changes is not comparable, additional qualification and/or bridging studies
21 should be undertaken to support the safety of the materials proposed for continued clinical
22 evaluation.

23
24 To ensure that appropriate stability data are collected during later stage clinical trials of the
25 adjuvanted vaccine, a stability protocol to be used for the formal stability studies should be
26 developed for the antigen, the adjuvant, and the adjuvanted vaccine. Stability programs should
27 be designed to monitor the chemical, physical, biological, and microbiological stability of the
28 antigen, the adjuvant, and the adjuvanted vaccine throughout the clinical testing program. The
29 properties of each of the antigen and adjuvant that are most indicative of stability, both when
30 stored individually and as a mixed final adjuvanted vaccine, should be identified as stability

1 evaluations proceed (as mentioned above in section 3.2.1). If it is determined that degradation
2 products accumulate from either the antigen or the adjuvant over the shelf-life of the adjuvanted
3 vaccine, these should be evaluated during stability testing of the final product and the NRA
4 should be consulted to determine whether additional suitable nonclinical toxicological testing
5 should be undertaken to confirm their safety. Additional guidance on stability testing of vaccines
6 can be found in the WHO Guidelines on the stability evaluation of vaccines (21).

8 **4. Rationale for the use of the adjuvant**

9 Adjuvant activity is a result of multiple factors and an adjuvant-mediated enhancement of the
10 immune response to one vaccine antigen, as a rule, cannot be extrapolated to the enhancement of
11 the immune response to another antigen. Individual antigens vary in their physical, biological
12 and immunogenic properties and antigens may have different needs for immunological help from
13 an adjuvant (5). Manufacturers should justify the choice of the adjuvant based on the immune
14 response desired, which may include effects on the magnitude, the breadth and/or the type of
15 immune response to specific antigens. In addition, adjuvants are also used in antigen dose-
16 sparing strategies with the aim of increasing the availability and supply of vaccines; for example,
17 under emergency situations of an influenza pandemic (22) or as a strategy to decrease the cost of
18 the vaccine (e.g., use of inactivated poliovirus vaccine for polio eradication) (23).

19
20 Many advances in the understanding of innate immunity have begun to provide insights into the
21 immunological mechanisms of adjuvant action. Many of the immunostimulatory adjuvants are
22 recognized by various members of the toll-like receptor (TLR) family, a subclass of pathogen-
23 recognition receptors, while other adjuvants may target other families of pathogen recognition
24 receptors that could prove to be important in shaping the adaptive immune response.

25 Furthermore, there are complex regulatory interactions between the many families of innate
26 receptors and other signaling pathways. Within this framework, the activities exerted by
27 adjuvants include, but are not limited to, the facilitation of (i) mobilization of antigen-presenting
28 and/or polymorphonuclear cells; (ii) antigen uptake and presentation of the antigen(s) in the
29 vaccine by antigen-presenting cells; (iii) secretion of proteins by antigen-presenting cells; (iv)
30 recruitment, targeting and activation of antigen-specific cells; (v) modulation of activities that

1 regulate the ensuing immune responses; and/or (vi) protection of the antigen from degradation
2 and elimination.

3
4 The scientific rationale supporting the benefit of adding the adjuvant and the choice of specific
5 adjuvant(s) should be provided by the adjuvanted vaccine manufacturer. Before evaluating a
6 particular adjuvant in combination with an antigen in a clinical trial, it is recommended that data
7 from *in vitro* and/or *in vivo* studies be generated to support the rationale for including the specific
8 adjuvant in the vaccine formulation and for selecting the dose range of adjuvant to be tested. In
9 the ideal case, the mode of action of the selected adjuvant as well as the mechanism of the
10 enhanced immune response would be well understood before late clinical development. When
11 the mode of adjuvant action is not well defined, supplemental *in vivo* or *in vitro* data (as
12 discussed in sections 4.1 and 4.2, respectively) may be provided, in addition to the pivotal
13 toxicity study, to support the added benefit of the adjuvant to the immune response induced by
14 the adjuvanted vaccine as well as the safety of the adjuvanted vaccine.

15

16 **4.1 *In vivo* proof of concept studies**

17 Data from proof of concept studies, including data from early studies conducted to evaluate
18 optimal antigen/adjuvant formulations, can provide important information with regard to the
19 characteristics of the adjuvanted vaccine. This includes evidence for the need for the adjuvant,
20 the type and magnitude of the immune responses induced (i.e., innate immunity, humoral and
21 cellular immunity), and their functional capacity to either protect against disease (i.e.,
22 prophylactic vaccine) or ameliorate an existing infectious disease (i.e., therapeutic vaccine) when
23 a relevant nonclinical disease model is available. These ‘pilot’ or exploratory studies designed to
24 identify and screen adjuvanted vaccine formulations may be non-GLP-compliant; however, they
25 may identify unknown or potential adverse effects and provide crucial information for the design
26 of GLP-compliant toxicity studies. In addition, such studies may provide the scientific
27 justification for manufacturing changes and for optimization of adjuvanted vaccine formulation,
28 dose, and route of administration during the clinical development of the adjuvanted vaccine
29 product.

30

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

- 5 1) Evaluation of humoral immune responses with regard to magnitude (e.g., mean titer or
6 concentration), quality (e.g., affinity or avidity), and functional activity (e.g., neutralizing
7 activity).
- 8 2) Evaluation of cellular immune responses including assessment of the induction of
9 specific types of cellular responses (e.g., examining Th1 or Th2 cytokine profiles or
10 testing for the induction of cytotoxic T cells).
- 11 3) Evaluation of protective or therapeutic responses against the relevant pathogen using
12 appropriate animal or *in vitro* disease models and/or evaluation of functional immune
13 responses (e.g., neutralizing activity, serum bactericidal or opsonophagocytic antibody
14 titers).
- 15 4) Evaluation of duration of (24), and extent of cross-protection provided by (25, 26) the
16 induced immune response.

17

18 These studies will contribute to the elucidation of the adjuvant mode of action and may provide
19 indication of the adjuvant-specific immune modulatory effects. In addition, these studies may
20 assist in the interpretation of nonclinical safety studies and the identification of potential adverse
21 effects to be monitored during clinical development. The development of *in vitro* model
22 systems, particularly those using human cells, is recommended also as they may provide
23 additional relevant information to elucidate the mechanism of action of the adjuvant (see section
24 4.2 below).

25

26 **4.2 *In vitro* supporting studies**

27 Functional *in vitro* bioassays may also provide helpful insight in understanding the mode of
28 action of a particular adjuvant and may provide valuable supplemental and complementary data
29 to the animal studies. This is important in particular when there are limitations of the animal
30 models such as species-specific differences (e.g., TLR receptors). Antigen-presenting cells or

1 other immune cells are widely used to assess and monitor the direct or indirect effects of
2 adjuvants by measuring activation parameters (such as changes in the expression of cell surface
3 molecules and the pattern of cytokine secretion), and more recently such human cells have been
4 used to develop *in vitro* assays that may be predictive of adjuvant safety *in vivo* (27). More
5 complex tissue culture systems containing a mixture of human immune cells mimicking
6 lymphoid tissue are being explored with the aim of evaluating human immune responses *in vitro*
7 (28).

8

9 **5. Considerations for selection of the animal species for nonclinical** 10 **evaluation of vaccine adjuvants and adjuvanted vaccines**

11 Investigations of the properties that influence the safety and pharmacological activity of the
12 adjuvant and the adjuvanted vaccine require the use of appropriate animal species. The animal
13 species used for pharmacological and safety evaluations should be chosen carefully and justified.
14 For ethical reasons, it is desirable to apply the 3R principles (reduction, replacement, refinement)
15 to the use of animals where scientifically appropriate (29). Both manufacturers and NCL/NRA
16 staff are encouraged to further develop *in vitro* assays and to evaluate their suitability for the
17 control of vaccines (30).

18

19 **5.1 Selection of animal species for nonclinical pharmacology studies**

20 For the purpose of this document the nonclinical pharmacological activity of an adjuvanted
21 vaccine is defined as the ability of the adjuvanted vaccine to induce and/or modify an immune
22 response in an animal species. Factors influencing the selection of a particular animal species
23 include, but are not limited to, the vaccine antigen, the adjuvant chosen, the type of immunity
24 (i.e., cell-mediated or humoral) to be induced and the route of administration. It is recommended
25 that proof-of-concept studies be undertaken using an animal species in which (i) an immune
26 response to the vaccine antigen is developed and, (ii) the immune response to the antigen is
27 enhanced by the adjuvant through a mechanism similar to that expected in humans (e.g., TLRs
28 known to be targeted by the adjuvant are present in the species, and enhanced humoral and/or
29 cellular immunity is observed). However, it is acknowledged that species-specific differences in
30 the immune responses induced in the animal species compared to the human are likely. Proof- of

1 concept studies most commonly are conducted in several animal species, including both naïve
2 and pre-exposed animals. In addition to evaluating the immune response induced by the vaccine
3 antigen alone and in the presence of adjuvant, the mechanism of action of the adjuvant in the
4 absence of the vaccine antigen should also be evaluated.

5
6 If the adjuvanted vaccine is a therapeutic vaccine for an infectious disease indication, where
7 feasible, disease animal models may need to be developed to study the pharmacologic activity of
8 the adjuvanted vaccine and its effect on the disease. For preventive adjuvanted vaccines, the use,
9 when available, of an animal species sensitive to the human pathogen, may provide important
10 insight into the mechanism of protection from the disease, e.g., the ferret model for human
11 influenza.

12
13 Nonclinical pharmacology studies may be conducted under non-GLP compliant conditions. It is
14 advisable to incorporate into the study design toxicological endpoints to guide the design of
15 GLP-compliant nonclinical safety studies. It is sufficient to conduct these studies in small
16 animal species if it can be demonstrated that the animal species chosen is relevant and responsive
17 to the vaccine antigen and the adjuvant when given by the intended route of administration.
18 Non-human primates should only be used if no other relevant animal species is available.

19

20 **5.2 Selection of animal species for nonclinical safety studies**

21 When selecting the animal species for the nonclinical safety studies, it is important to document
22 the pharmacological activity of the vaccine in the presence and absence of adjuvant in that
23 species. It is recommended that manufacturers conduct nonclinical safety studies in compliance
24 with GLP (see Table 1 and section 6.2) and using an animal species in which an immune
25 response to the vaccine antigen is developed and, ideally, the immune response to the antigen is
26 enhanced by the adjuvant through a similar mechanism as expected in humans. It is not
27 necessary, however, to conduct the nonclinical safety study in the same animal species used for
28 proof of concept or nonclinical pharmacology studies (see sections 4.1 and 5.1). Non-human
29 primates should only be used if no other relevant animal species is available. In situations where
30 no animal species is available that is responsive to the adjuvanted vaccine, the choice of the
31 animal species should be justified. In some circumstances, use of *in vitro* model systems,

1 particularly those using human cells, to evaluate the toxicity of the adjuvanted vaccine may
2 provide additional supplementary information to assist in interpreting toxicity data (27).

3

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

11

12 The route of administration used in the toxicity study should correspond to that intended for use
13 in the clinic. Also, when the adjuvanted vaccine is to be administered in the clinic using a
14 particular device, the same device should be used in the animal study, where feasible. For
15 example, a small rodent species may not be an appropriate choice for nonclinical evaluation of a
16 vaccine that is to be delivered intranasally because some of the inoculum could be delivered to
17 the lungs. In this case, a larger animal or one with nasal surface area, anatomy, and physiology
18 similar to that of humans would be more appropriate (31).

19

20 Use of a single species is generally acceptable (see section 6.2). This approach has commonly
21 been accepted based primarily on pragmatic considerations, for example, the ability to predict
22 the human immune response may be limited due to species-specificity of the response in animals
23 to the antigen, the adjuvant, or both.

24

25 **5.3 Limitations of animal studies**

26 The limitations of using animals to characterize the pharmacologic and safety profile of an
27 adjuvant or adjuvanted vaccine are acknowledged. The ability to predict the human immune
28 response based on pharmacologic studies in an animal may be limited due to the species-
29 specificity of the response to the antigen, the adjuvant or both. Similarly, local and systemic
30 adverse effects observed in a nonclinical safety study may not be directly translatable to the

1 clinic. In addition, rare and/or late onset adverse events that may occur in human subjects as a
2 result of adjuvanted vaccine administration may not be observed in animal studies.
3 Nevertheless, these studies offer the best currently available tools to evaluate the preclinical
4 safety and pharmacology of adjuvanted vaccines.

5

6 **6. Nonclinical safety assessment in animals**

7 **6.1 General remarks**

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

15

16 Safety assessments in animals studies are valuable tools to help define an acceptable
17 adjuvant/antigen ratio and a safe dose, as well as to identify unknown or potential adverse effects
18 that should be taken into consideration for further product development or to be monitored in
19 future clinical trials. The type of studies and the timing in relation to the clinical program are
20 presented in below in section 6.2.

21

22 **6.2 Toxicity studies of vaccine adjuvants and final adjuvanted vaccine** 23 **formulations**

24 The preclinical toxicity studies of the final adjuvanted vaccine formulation should be adequate to
25 identify and characterize potential adverse effects of the vaccine in order to conclude that it is
26 reasonably safe to proceed to first-in-human clinical investigation. As the mechanism of action
27 of the adjuvant and/or adjuvanted vaccine formulation is often not fully understood, the toxicity
28 studies should be designed to evaluate a broad spectrum of parameters due to the uncertainty of
29 the *in vivo* effects and associated outcomes. Toxicity studies should be designed to mimic the
30 intended route of administration in the clinic and to evaluate local reactogenicity (e.g., injection

1 site inflammation) and systemic toxicity (i.e., toxicity that is occurring at sites distant from the
2 site of initial application). Pivotal toxicity studies should use the intended final formulation and
3 dose of the adjuvanted vaccine (see section 3.2) and should be conducted in compliance with
4 GLP.

5
6 When properly designed, conducted, and interpreted, and when no major safety signals are
7 revealed in the study results, one repeated dose toxicity study in one relevant species should be
8 sufficient. However, if there are significant manufacturing or formulation changes during
9 product development, additional animal toxicity studies may be recommended to confirm that
10 the safety profile of the product has not been changed. Also, throughout the clinical program,
11 additional animal toxicity studies may be necessary to investigate any adverse events observed in
12 clinical trials or to support the use of the vaccine in a special population (e.g. developmental and
13 reproductive toxicity studies).

14
15 While comprehensive toxicity evaluations of the final adjuvanted vaccine formulation are
16 considered essential, the advantages and limitations of toxicity studies with adjuvant alone have
17 been discussed extensively in previous meetings and workshops (7-11). A comprehensive
18 toxicity assessment of the adjuvant alone in animals (or of individual evaluations of its multiple
19 components, if a combination adjuvant) may not be needed as a separate program. However, to
20 enable the interpretation of immunogenicity and safety studies of the adjuvanted vaccine, a study
21 arm receiving adjuvant alone may be included in the repeated dose toxicity studies (see sections
22 below) that are part of the comprehensive toxicity evaluations of the final adjuvanted vaccine
23 formulation.

24 25 ***6.2.1 Safety pharmacology studies***

26 The purpose of safety pharmacology study is to investigate the effects of the candidate vaccine
27 on vital functions. Although not usually required, safety pharmacology studies may be
28 recommended by the NRA in some cases. For example, if data from nonclinical and/or human
29 clinical studies suggest that the adjuvanted vaccine may affect physiological functions (e.g.,
30 central nervous system, respiratory, cardiovascular, renal functions and body temperature) other

1 than the immune system, safety pharmacology studies should be incorporated into the safety
2 assessment program.

3

4 **6.2.2. Repeated dose toxicity studies**

5 This section highlights important considerations regarding the study design for pivotal toxicity
6 studies that should be conducted with the same vaccine formulation intended to be used in
7 clinical trials (see section 3.2). If more than one dose of antigen or adjuvant is to be evaluated in
8 the clinical study, the formulation containing the highest dose (i.e., “worst case”) should be
9 included in the pivotal toxicity studies. Single dose toxicity studies on the final formulated
10 vaccine product, which are applicable to small molecule chemical drugs, are usually not needed
11 in the accordance with ICH M3(R2) (32). Acute effects of administering the vaccine can also be
12 monitored in repeated dose toxicity studies if they are adequately designed (e.g., evaluation is
13 conducted after the first administration). Alternatively, acute effects can be assessed in a single
14 dose design as part of a local tolerance study. For a study intended to support a first-in-human
15 clinical trial, the number of animals studied per sex, group, and time interval should be sufficient
16 to allow meaningful scientific interpretation of the data generated. The size of the treatment
17 group will depend on the animal species chosen, i.e., the number of animals included in studies
18 using non-rodents (e.g., miniature pigs) would be expected to be less than the number included in
19 studies using rodents. For mice and rats, it is recommended that at least ten animals/sex/group be
20 used for the necropsy at the end of the treatment interval and at least five animals/sex/group be
21 used for the necropsy at the end of the recovery period. For rabbits, it is recommended that at
22 least five animals/sex/group/time interval be used. In general, the approximate age for rodents
23 should be 6 to 8 weeks, and for rabbits, 3 to 4 months, at the start of the study.

24

25 **6.2.2.1 Dose, dosing regimen, and controls**

26 Dose response evaluation for the adjuvanted vaccine is generally not required as part of the basic
27 toxicity assessment, given that, in most cases, dose response assessment was explored in
28 nonclinical pharmacology studies. For adjuvanted vaccines, the toxicity study should be
29 performed using the highest anticipated human dose (in absolute terms) of the final adjuvanted
30 vaccine to be used in the proposed clinical trial, where feasible. Ideally this dose provides

1 optimal exposure of the animal to the candidate vaccine and the immune response induced.
2 However, in the case of a novel adjuvant, it may be advisable to include additional (lower and
3 higher) doses of the adjuvanted vaccine formulation or adjuvant alone in order to identify a safe
4 dose that could be used in a first-in-human clinical trial.

5
6 If the dose to be administered is limited by the total volume that can be administered in a single
7 injection, guidelines for animal welfare should be followed (33). In such cases, the total volume
8 may need to be administered at multiple sites using the same route of administration; however, it
9 should be noted that the evaluation of local reactogenicity might be less reliable in such cases.

10
11 For adjuvanted vaccines intended to be given repeatedly, the number of doses administered to
12 the animals in repeated dose toxicity studies should equal or exceed the number of doses
13 proposed in humans. However, in many cases, the studies are designed to include one dose more
14 than planned for the clinical trial to allow for the possible inclusion of an additional dose in the
15 clinical trial. To simulate the proposed clinical usage, vaccine doses should be given as episodic
16 doses, but the dosing interval used in the toxicity study may be reduced (e.g., 2 to 3 weeks
17 interval) compared to the proposed clinical dosing interval (which usually is greater than 2 to 3
18 weeks). The nonclinical dosing interval should primarily be based on the kinetics of the primary
19 and secondary antibody response observed in the animal study.

20
21 In general, the study design should include a negative control group that receives an inert
22 placebo such as saline to evaluate a baseline level of treatment and an adjuvant alone arm to aid
23 in the interpretation of safety data from the adjuvanted vaccine. Also, the study should include
24 additional treatment groups to be sacrificed and evaluated as described below at later time points
25 after treatment, to evaluate reversibility of adverse effects observed during the treatment period
26 and to detect potential delayed adverse effects.

27

28 **6.2.2.2 Route of administration**

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

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6.2.2.3. End-points in toxicity studies

The following section discusses endpoints which 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.

6.2.2.3.1 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 post-mortem data should be collected. This routinely includes careful monitoring of body weight and food consumption, body temperature, histopathology, clinical chemistry, hematology, 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., clinical observations, body weights, 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 a week). Body temperature should also be evaluated prior to, and 3 to 8 and 24 hours after each dose. If there is an increase in temperature, additional measurements should be taken every 24 hours until the values return to baseline. Interim analysis of haematology and serum chemistry should be considered within approximately 1 to 3 days following the first and last dose administration and at the end of the recovery period; in addition, the collection of a pre-dosing sample is

1 recommended. Coagulation parameters should be included routinely; in some cases, evaluation
2 of urine samples and serum immunoglobulin classes may be of value. Additionally, it is
3 recommended that species appropriate acute phase reactants (e.g., C-reactive protein) be
4 measured in the toxicity study prior to immunization, at time points following the administration
5 of the adjuvant or adjuvanted vaccine that have been demonstrated to reflect peak elevations in
6 the acute phase reactants being evaluated (commonly 24 to 48 hours), and after a recovery phase
7 of 7 days. When measuring acute phase reactants, the choice of the animal species may
8 determine which proteins can be measured as these reactants vary among species (34). The data
9 discussed above should be collected not only prior to and during the treatment phase, but also
10 following the treatment free (recovery) phase (i.e., 2 or more weeks following the last dose) to
11 determine persistence, exacerbation and/or reversibility of potential adverse effects.

12
13 Post-mortem data including gross necropsy (with tissue collection and preservation, including
14 gross lesions and organ weights) should be collected within 3 days following the last dose and
15 following the above-mentioned recovery period (e.g., 2 or more weeks following the last dose)
16 (1). At study termination, final body weights (following overnight fasting) should be obtained.
17 Terminal blood collection and analysis should include serum chemistry, haematology, and
18 coagulation parameters as well as an immune response evaluation. Histopathological
19 examinations should always include pivotal organs (brain, lung, heart, kidneys, liver,
20 reproductive organs) and the site of adjuvant or adjuvanted vaccine administration. Special
21 attention should be paid to the immune organs, i.e., lymph nodes (draining and distant to
22 application site), thymus, spleen, bone marrow and Peyer's patches or bronchus-associated
23 lymphoid tissue, as well as organs that may be primarily affected due to the particular route of
24 administration. The extent of the list of tissues to be examined (i.e., the full tissue list as
25 provided in Appendix 2 vs. the reduced list mentioned above limited to immune and pivotal
26 organs) will depend on the adjuvant or adjuvanted vaccine in question, as well as on the
27 experience and knowledge obtained through previous nonclinical and clinical testing of the
28 vaccine components. Additionally, any known target organs of the adjuvant or adjuvanted
29 vaccine should be evaluated. For novel adjuvants and adjuvanted vaccines containing a novel
30 adjuvant, it is recommended that the full tissue list be evaluated.

31

1 *6.2.2.3.2. Parameters for monitoring of local reactogenicity*

2 Local toxicities should be determined at the site(s) of adjuvant or adjuvanted vaccine
3 administration and any other sites that come into contact with the adjuvant or adjuvanted vaccine
4 components as a result of the method of administration. Local toxicity with intramuscularly
5 administered vaccines should preferably be conducted in animals with sufficient muscle mass to
6 test the full human dose of the final vaccine formulation. However, in some cases, the dose is
7 limited by the total volume that can be administered in a single injection; in such cases, the total
8 volume may be administered at more than one site using the same route of administration, and
9 injection sites should be collected and evaluated for local toxicity.

10

11 Injection site reaction after inoculation should be scored using a prospectively defined system
12 (e.g., the modified Draize test (35)), along with an assessment of any vesiculation, ulceration,
13 severe eschar formation, and other manifestations of significant toxicity, e.g., limb impairment.

14

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

21

22 ***6.2.3. Developmental and reproductive toxicity***

23 As vaccination programs may include women of childbearing potential, it is important to
24 consider the need for developmental and reproductive toxicity studies. As is the case for general
25 toxicity, the use of a novel adjuvant may require adding an adjuvant-alone arm in the
26 reproductive toxicity studies. However, the study design is also dependent on the intended
27 clinical use of the vaccine. For example, vaccination may be given early in pregnancy to protect
28 the mother at risk, or might be given later in pregnancy to induce passive immunization to
29 protect the infant directly from birth. Depending on the adjuvant, there may be concern about an
30 adjuvant-induced systemic inflammatory response, e.g., fever, which may adversely affect early

1 pregnancy (e.g., implantation or placental growth) (36). In these cases, it is recommended to
2 include in the study design an additional treatment group to evaluate the effect of adjuvant on
3 early pregnancy parameters. Rather than dosing this treatment arm prior to mating, it is
4 recommended to dose animals post-mating and prior to implantation, e.g., post-mating day one.
5

6 In general, the administration of one or several additional doses during organogenesis (i.e.,
7 implantation to closure of the hard palate) is recommended in order to evaluate potential direct
8 embryotoxic effects of the components of the vaccine formulation, and, depending on the animal
9 model, to allow maternal antibody transfer to the fetus during pregnancy or the lactation period.
10 Endpoints in the embryofetal/peri-postnatal toxicity studies include, but are not limited to,
11 viability, abortions, number of resorptions, fetal body weight, morphology, pre-weaning
12 development and growth as well as survival incidence and developmental landmarks. For details
13 on such studies, please see the *FDA Guidance for Industry: Considerations for Developmental*
14 *Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications* (37)
15 and the *WHO Guidelines on Nonclinical Evaluation of Vaccines* (1).
16

17 In most cases, the developmental and reproductive toxicity studies can be performed in parallel
18 to the clinical trials. However, some NRAs require that women of child-bearing potential be
19 excluded from large scale, late stage clinical trials that are conducted prior to the completion of
20 developmental and reproductive toxicity studies; other NRAs require the use of appropriate birth
21 control methods for women of child bearing potential that are included in clinical trials. Further
22 considerations can be found in ICH M3(R2) (32).
23

24 **6.2.4 Biodistribution studies**

25 Adjuvants are expected to exert their action locally in close connection to the antigen. However,
26 biodistribution studies can be helpful in understanding the distribution of the adjuvant following
27 injection. The feasibility of and need for such biodistribution studies should be evaluated on a
28 case by case basis.
29

1 **6.2.5 Genotoxicity/carcinogenicity studies**

2 Genotoxicity studies are normally not needed for the final vaccine formulation (1). However, a
3 standard battery of genotoxicity studies is generally recommended for most novel adjuvants that
4 are (or contain) new chemical entities (32, 38). Based on previous experience, carcinogenicity
5 studies are generally not needed for adjuvants or adjuvanted vaccines.

7 **6.2.6. Toxicity studies of adjuvant alone**

8 As noted above (section 6.1), comprehensive toxicity assessment of the adjuvant alone in
9 animals may be included as part of the study design with the adjuvanted vaccine (see 6.2).
10 However, evaluation of the adjuvant alone can be important for novel adjuvants that have not
11 been studied previously or will be used in multiple different vaccine formulations. In the case of
12 a novel adjuvant or combination adjuvant, it may be advisable to include additional (lower and
13 higher) doses of the adjuvant component(s) in order to identify a safe dose that could be used in a
14 first-in-human clinical trial and, as well as safety signals that should be monitored in the
15 proposed clinical trial.

16
17 Although not usually required, safety pharmacology studies may be recommended in some cases
18 to demonstrate that a novel adjuvant has no adverse effects on physiological functions (e.g.,
19 central nervous system, respiratory, cardiovascular, renal functions, and body temperature) other
20 than the immune system. If needed, such evaluations could also be included as a specific arm
21 with the adjuvant alone in the repeated-dose toxicity study of the intended final vaccine
22 formulation (1, 39). It is expected that these studies would be conducted before initiating first-
23 in-human clinical trials.

25 **6.2.7 Summary of recommendations regarding timing of studies**

26 In general, the guidance provided in this document regarding the timing of studies in relation to
27 clinical trials is consistent with that of other guidance documents, e.g., *ICH Guidance on*
28 *Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing*
29 *Authorization for Pharmaceuticals, M3(R2)* (32). A repeated dose toxicology study (including
30 safety pharmacology endpoints, if needed) should be conducted before the first-in-human clinical

1 trial. It may be important to conduct some studies with adjuvant alone (e.g., systemic toxicity
2 and genotoxicity, where needed) prior to initiation of clinical trials (32). Developmental
3 toxicology studies should be performed prior to initiation of any clinical study to be conducted in
4 pregnant women, i.e., for those vaccines specifically developed for use in pregnancy. For
5 vaccines indicated for females of childbearing potential, subjects can be enrolled in clinical trials
6 provided that appropriate precautions are taken to avoid vaccination during pregnancy, such as
7 pregnancy testing and use of birth control. For these products, developmental toxicity studies
8 (section 6.2.3) may be performed in parallel with the clinical study. All nonclinical safety
9 studies should be completed by the time a license application is submitted to the NRA.

11 **6.3 Additional considerations**

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

22
23 Thus far, there is no compelling clinical evidence that adjuvants are causally related to the
24 induction of autoimmune phenomena (or autoimmune disease) or hypersensitivity in humans (4).
25 Although there has been interest in developing animal models that could be used to screen
26 adjuvants and adjuvanted vaccines for induction of autoimmunity or hypersensitivity, such
27 models do not currently exist. Therefore, no recommendations can be made at this time
28 regarding specific nonclinical studies that should be conducted. These are complex and multi-
29 factorial conditions; further research is needed to identify additional biomarkers related to
30 autoimmunity and hypersensitivity phenomena.

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7. 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 unadjuvanted vaccines (2); however, some issues unique to clinical evaluation of vaccines with novel adjuvants may need to be considered. The initial clinical trials of adjuvanted vaccines are usually intended to i) 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 endpoint trials and ii) 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 toxicologic 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 in support 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

1 including relevant challenge models when available) conducted prior to the initiation of clinical
2 trials. In addition, it is recommended that the early clinical evaluations of an adjuvanted vaccine
3 be designed to include the evaluation of both antigen alone and adjuvanted vaccine arms to
4 demonstrate the added benefit of the adjuvant; such data may include, for example, evidence of
5 enhanced immune responses or antigen sparing.

6
7 If the safety of the adjuvanted vaccine was evaluated in appropriately designed toxicology
8 studies that were conducted in line with the recommendations outlined above and if there were
9 no adverse effects observed in the toxicology studies conducted, the human dose tested in the
10 toxicology studies may be acceptable as the starting dose in the first-in-human trials. However,
11 such clinical trials are often designed as dose-escalating studies where the antigen and/or the
12 adjuvant are given at escalating doses. With this in mind, given the limitations of the animal
13 studies, it may be prudent to consider using a safety factor (for example, a safety factor of 10 has
14 been used historically) and to divide the human dose tested in the toxicology studies by the
15 safety factor to find the recommended starting dose and escalate the dose from there. While it is
16 anticipated that the adjuvant may have an antigen-sparing effect, the first-in-human trials should
17 be designed to attempt to establish whether the adjuvant is needed and if so, the minimum dose
18 of adjuvant that is necessary to achieve adequate immunogenicity.

19
20 Although an inactive control (placebo) group may not be required in the first-in-human trial of
21 an adjuvanted vaccine, the inclusion of a group receiving inactive control, such as inert saline
22 placebo, in early phase clinical trials will enhance interpretation of the initial safety data through
23 control for placebo effects and circulating community acquired illnesses. The inclusion of an
24 adjuvant alone arm should be discussed with the relevant NRA as some regulatory authorities
25 recommend that such arms be avoided for ethical reasons; in those cases, an antigen alone
26 control arm may be preferred.

27
28 As with first-in-human trials of non-adjuvanted vaccines, those for adjuvanted vaccines are
29 usually conducted in a limited number (e.g., 20-80) of healthy, adult volunteers (e.g., 18-50 years
30 of age) with safety as the primary objective. To aid in the overall risk/benefit evaluation of the
31 adjuvanted vaccine, the subject population should be clearly defined by inclusion and exclusion

1 criteria, and the subjects should be closely monitored for safety. The clinical protocol should
2 contain a safety monitoring plan with details regarding active post-vaccination monitoring and
3 pre-defined toxicity criteria for assessing the severity of clinical and laboratory parameters (40).
4 In addition, the plan for increasing the dose of antigen and adjuvant, with pre-defined step-wise
5 criteria for doing so, should be included in the clinical protocol. Also, it is recommended,
6 especially when a novel adjuvant is used, that safety monitoring be extended through 12 months
7 following the last vaccination (where the last follow-up may be accomplished by a telephone
8 call). In this regard, it is recommended that serum specimens be banked where possible for
9 potential future assessment in the event of a serious adverse event (SAE), a new-onset medical
10 condition, or an adverse event of special interest that develops later on in the course of the first-
11 in-human clinical trial.

12
13 Any safety experience with the same adjuvant formulated with other vaccine antigens, if
14 available, may assist in developing the safety monitoring plan for the adjuvanted vaccine.
15 However, since the mode of action in humans for the adjuvant in the specific adjuvanted vaccine
16 to be evaluated in the first-in-human trial is usually unknown and adjuvants may exhibit a range
17 of properties that invoke complex immune responses, it is recommended that first-in-human
18 trials for adjuvanted vaccines include inquiries regarding specific adverse events. This may
19 include, for example, local reactions (e.g., pain, redness, swelling, granuloma formation, abscess,
20 necrosis; and regional lymphadenopathy); systemic reactions (e.g., fever, nausea, diarrhea, and
21 malaise); immune-mediated toxicity (e.g., cytokine release, immune suppression, and
22 autoimmune disease); and teratology. Examples of adverse events of “special interest” may
23 include neuroinflammatory disorders (e.g., optic neuritis and transverse myelitis),
24 musculoskeletal and connective tissue diseases (e.g., rheumatoid arthritis, systemic lupus
25 erythematosus (SLE), and Wegener’s granulomatosis), and gastrointestinal disorders (e.g.,
26 Crohn’s disease and ulcerative colitis). Additionally, targeted laboratory assessments [e.g., C-
27 reactive protein, fibrinogen, antinuclear antibody (ANA), anti-neutrophil cytoplasmic antibodies
28 (ANCA), and rheumatoid factor] may aid in the evaluation of adverse events and medical
29 conditions.

30

1 **Table 1. Summary of manufacturing and quality information**
 2 **needed for pharmacology studies, nonclinical toxicology studies ^a**
 3 **and first-in-human trials**

Comment on Manufacturing and Quality Information	Pharmacology	Toxicology ^a	First-in-human trials
Raw Materials Ideally, they should be the same throughout nonclinical pharmacology studies, toxicology studies and first-in-human trials.	Purity and source important	Purity and source important	Purity, source and control of manufacture important
Production of Intermediates and Adjuvanted Vaccines	Small scale	Small scale. Ideally, should be the same lots as those for first-in-human trials (or comparable to lots for first-in-human trials in terms of manufacturing process and controls)	Small scale, but should be manufactured in compliance with the appropriate GMP
Presentation	Often provided in separate containers to be mixed prior to use	May be provided as an already mixed formulation (“adjuvanted vaccine”) or provided in separate containers to be mixed prior to administration	May be provided as an already mixed formulation (“adjuvanted vaccine”) or provided in separate containers to be mixed prior to administration
Characterization	May be less extensive characterization. Usually general quality (e.g., composition, purity, potency ^b)	Should be considerable characterization of material (e.g., purity, physicochemical data, potency ^{b,c}). Stability should be assessed.	Should be considerable characterization of material (e.g., purity, physicochemical data, potency ^c). Stability should be assessed.

- 1 ^a Should be compliant with Good Laboratory Practices (GLP).
- 2 ^b Potency of the adjuvanted vaccine should be considered, if a potency assay has been
3 developed. Testing of the adjuvant identity and content is recommended.
- 4 ^c If the adjuvanted vaccine is provided pre-mixed in one container, the potency of the
5 adjuvanted vaccine formulation should be determined. For some adjuvanted vaccines (e.g.,
6 aluminum-adsorbed vaccines), it may not be possible, depending on the nature of the potency
7 assay, to evaluate the potency of the final formulated vaccine by certain assays. In this case,
8 the determination of potency of the antigen alone prior to adsorption may be recommended
9 as well as the development of an *in vivo* method for potency assessment of the adjuvanted
10 vaccine.
- 11

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1 **Appendix 1: Examples of adjuvant classes**

2

3 The following list identifies the main classes of adjuvants either in current use in licensed
4 vaccines or under evaluation. It is an updated version of the list of adjuvants (1) that was
5 provided by the Committee for Medicinal Products for Human Use (CHMP); for each category,
6 representative examples are provided for illustration.

7

8 Classification of adjuvants

- 9 • Mineral salts/gels, e.g., aluminium hydroxide and aluminium or calcium phosphate gels.
- 10 • Oil-in water and water-in-oil emulsions, amphiphilic molecules and surfactant based
11 formulations, e.g., MF59 (microfluidised detergent stabilised oil-in-water emulsion), QS-
12 21 (purified saponin, which is plant-derived), AS03 (consisting of an oil-in-water
13 emulsion plus alpha-tocopherol), Montanide ISA-51, and Montanide ISA-720.
- 14 • Particulate adjuvants, e.g., liposomes, virosomes (unilamellar liposomal vehicles
15 incorporating influenza haemagglutinin), ISCOMS (structured complex of saponins and
16 lipids), and polylactide co-glycolide (PLG), PLG -Dimethylaminoethane-carbamoyl-
17 Cholesterol (PLGA/DC-cholesterol) particles, and Iscomatrix.
- 18 • Microbial derivatives (natural and synthetic), e.g., monophosphoryl lipid A (MPL),
19 Detox (MPL + M. Phlei cell wall skeleton), AGP [RC-529] (synthetic acylated
20 monosaccharide), DC_Chol (lipoidal immunostimulators able to self-organise into
21 liposomes), OM-174 (lipid A derivative), CpG motifs (synthetic oligodeoxynucleotides
22 containing immunostimulatory CpG motifs), bacterial flagellin genetically fused with
23 antigen, modified heat labile enterotoxin (LT) and cholera toxin (CT) (genetically
24 modified bacterial toxins that have been genetically modified to provide non-toxic
25 adjuvant effects); synthetic dsRNA, Poly IC:LC (Hiltonol) and Poly I: Poly C₁₂U
26 (Ampligen®).
- 27 • Endogenous human immunostimulators, e.g., hGM-CSF or hIL-12 (cytokines that can be
28 administered either as protein or plasmid encoded), Immudaptin (C3d tandem array).
- 29 • Inert vehicles, e.g., gold particles.
- 30 • Inert polysaccharides, e.g., Advax (delta-inulin), derived from plants (dahlias).

- Combination adjuvants or adjuvant systems that usually consist of combinations of vaccine delivery systems and immunostimulatory agents and may result in more effective delivery of the immunostimulatory adjuvant as well as the antigen, e.g., AS01 consisting of liposomes, MPL, and QS-21; AS02 consisting of an oil-in-water emulsion plus MPL and QS-21; AS03 consisting of an oil-in-water emulsion plus alpha-tocopherol; AS04 consisting of MPL and aluminum hydroxide; AS15 consisting of liposomes, MPL, QS-21 and a CpG oligodeoxynucleotide; and GLA-SE consisting of a synthetic acylated monosaccharide in a stable oil in-water emulsion.

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1 **Appendix 2: List of tissues (depending on the species) to be collected**
 2 **in a repeated dose toxicity study^a**

3

adrenal glands	nasal-oro-pharyngeal cavity (depending on vaccine and adjuvant)
aorta (thoracic)	oesophagus
bone (femur) with articulation	optic nerves
bone (sternum) with bone marrow	ovaries
bone marrow smears ^b	oviducts
brain	pancreas
bronchi (main-stem)	parathyroid glands
caecum	Peyer's patches
colon	pituitary gland
diaphragm	prostate
duodenum	rectum
epididymides	salivary glands (mandibular, parotid, sublingual)
eyes	sciatic nerves
gall bladder	seminal vesicles
Harderian glands	skeletal muscle
heart	skin
ileum	spinal cord (cervical, thoracic, lumbar)
injection site(s) (a sample should be taken covering the area of injection)	spleen
jejunum	stomach
kidneys	testes
lachrymal glands (main body and subconjunctival part)	thymus
larynx	thyroid glands
liver	tongue
lungs	trachea
lymph nodes (draining the injection site(s))	ureters
lymph nodes (non-draining the injection site(s), e.g., mandibular, mesenteric)	urinary bladder
nasal tissue (skull/nasal cavity)	uterus (body + horns + cervix)
mammary gland	vagina
	tissues with macroscopic observations

^a The above list of tissues is a comprehensive list of tissue to evaluate for local and systemic toxicity in repeated dose toxicity studies with some additional tissues included to represent adjuvanted vaccine specific targets. It represents an updated version of the list that was provided originally for vaccines (1) and then broadened and harmonized by the CHMP (2) and the Society of Toxicologic Pathologists (3).

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

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