

Annex 1

Guidelines for assuring the quality and nonclinical safety evaluation of DNA vaccines

This document provides information and guidance to national regulatory authorities and vaccine manufacturers concerning the characteristics, production, control and nonclinical development of DNA vaccines. The text is written in the form of Guidelines instead of Recommendations because further work is still needed to develop and standardize appropriate methods and criteria that will assure the consistent quality, safety and stability of these vaccines. Guidelines allow greater flexibility than Recommendations with respect to expected future developments in the field and indicate present deficiencies.

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1. Introduction

Vaccination involves priming the immune system of a host with an infectious agent or components of an infectious agent modified in a manner to ensure that the vaccine does not cause any harm or disease to the host, but ensures that when the host is confronted with that infectious agent, its immune system can respond adequately control the invading organism before it causes any ill effect. For over a hundred years immunization has been achieved by one of two basic approaches:

- introducing into the host, specific antigens against which the immune system will react directly; or
- introducing attenuated living organisms which replicate within the host without causing disease and synthesize the appropriate antigens which subsequently prime the immune system.

Since the early 1990s a radically new approach to vaccination has been actively and vigorously developed. This involves the direct introduction of plasmid DNA containing the gene encoding the antigen against which an immune response is sought into appropriate host tissues and the *in situ* production of the target antigen(s). This approach offers a combination of potential advantages over the more traditional approaches, including the stimulation of both B and T cell responses, improved stability of the vaccine, absence of any infectious agents and the relative ease of large scale manufacture. Many scientific publications address the potential of DNA vaccination and immune responses in animal models have been obtained using genes from a variety of infectious agents including influenza virus, hepatitis B virus, human immunodeficiency virus, rabies virus, lymphocytic choriomeningitis virus, West Nile virus, malaria and mycoplasma. In many cases protection from disease in animal models has also been demonstrated and many aspects of the immune response generated by the injection of plasmid DNA vaccines have been revealed although much remains to be understood. The value and advantages of DNA vaccines will be assessed on a case-by-case basis and their applicability will depend on the nature of the organism being immunized against, the nature of the antigen and the type of immune response required for protection.

DNA vaccines progressed rapidly into phase I clinical trials. However, the immune responses observed in animal models have generally not been reproduced in humans and many approaches have been and are being followed to enhance the human immune response. These approaches function in different ways such as in enhanced uptake, stability of expression, modulation of the immune response, or in adjuvanting, and include:

- complexing the DNA with polymers (enhances uptake, improves stability);

- encapsulating the DNA on or within microparticles (assists uptake, presentation and stability);
- optimization of the codon usage of the gene encoding the antigen of interest (enhances expression);
- encoding a variety of T cell epitopes either instead of or in addition to a full size protein antigen (modulates the immune response by targeting T cell stimulation);
- optimizing administration, e.g. by particle-mediated delivery (gene gun) or electroporation (enhances uptake, modulates immune response);
- route of administration, e.g. mucosal versus parenteral (modulates the immune response);
- boosting with viral vectors or protein antigen following an initial priming with plasmid DNA (improves immune response); and
- co-administration of DNA encoding an immune stimulatory molecule (molecular adjuvant), e.g. a cytokine (improves immune response, modulates the immune response).

Other approaches may also be under development now or in the future. The above approaches to enhancing the efficacy of a DNA vaccine may raise specific safety concerns and these should be addressed in appropriate nonclinical safety testing.

DNA vaccines are also being developed for veterinary use and efficacy in animal target species is being observed in some trials. Potentially protective immune responses are being observed against many infectious agents in several target species including fish, and companion and farm animals. A DNA vaccine against West Nile virus for use in horses was first licensed in the USA in 2005. Although the quality and safety considerations for vaccines for veterinary use differ from those for human use, experience with veterinary DNA vaccines can provide valuable information for the control and use of human DNA vaccines.

These guidelines concentrate on the quality control and on nonclinical testing of vaccines based on bacterial plasmid DNA intended for use in humans. The purpose of this document is to provide guidance on:

- appropriate methods for the control of the manufacture and characterization of plasmid DNA vaccines;
- appropriate approaches for the nonclinical testing of plasmid DNA vaccines; and
- information specific to plasmid DNA vaccines that should be included in submissions by manufacturers to national regulatory authorities in support of applications for the authorization of clinical trials and for marketing.

The development and application of DNA vaccines continues to evolve. Since these guidelines were first adopted in 1996 (1), many clinical trials of

DNA vaccines have taken place and much experience in their manufacture and control has accrued. This revision reflects the experience gained, especially in relation to the data derived from nonclinical safety testing and the concerns expressed in the first version of these guidelines. The control of these vaccines should continue to be approached in a flexible manner to enable further modifications as more experience is gained in their production and use. The intention is to provide a scientifically sound basis for the manufacture and control of these vaccines for use in humans so as to ensure their consistent safety and efficacy. Individual countries may wish to use this document to develop their own national guidelines for DNA vaccines.

2. **Scope of the guidelines**

This document provides guidance on quality and nonclinical aspects of DNA vaccines intended for use in humans.

The active constituent of a DNA vaccine is a plasmid molecule that contains the gene for a component of a pathogenic organism under the control of a mammalian expression system, and possesses DNA sequences necessary for replication and selection in bacteria. Although a vaccine is generally defined as a biological medicinal product for the prophylaxis of infectious disease, “DNA vaccines” are also being developed for therapeutic use, either against infectious disease or for other diseases such as cancer. In the case of cancer, the relevant gene often has a human origin (e.g. a cytokine) rather than a microbiological origin. DNA vaccines against infectious disease may also contain plasmids expressing genes of human origin which act as molecular adjuvants. It is clear that the manufacture and quality control of plasmid DNA for any of the above indications will be essentially identical and consequently, these guidelines are applicable to DNA vaccines for therapeutic as well as prophylactic use. The detailed design of relevant nonclinical safety testing should take into account the proposed use of the DNA vaccine and the risk–benefit situation.

The guidelines cover DNA vaccines regardless of their method of delivery.

The quality section of these guidelines will be applicable to DNA plasmids that contain mammalian viral replicons; however, different requirements may apply to nonclinical testing of such products and the present guidelines do not address these. Similarly, many aspects of the guidelines may be applicable to vaccines based on RNA, although again, different requirements are likely to apply especially for nonclinical safety testing for these types of vaccine. Plasmid DNA vaccines for use in gene therapy, DNA vaccines derived in eukaryotic cells, vaccines in which a bacterial cell acts as a carrier for a plasmid DNA encoding a relevant antigen and nucleic acid vaccines

made entirely by chemical means such as synthetic oligonucleotides are all outside the scope of these guidelines.

The quality section addresses the control of the bulk purified plasmid (drug substance) including control of the manufacturing process and the starting materials, characterization of the purified plasmid and control of the final formulated vaccine (drug product) including formulation, the control of materials used in formulation and characterization of the final vaccine.

The nonclinical safety section addresses the approaches to be followed during testing of the vaccine prior to clinical use. The background information provided in the quality section should be considered when designing appropriate nonclinical safety studies.

In general, recommendations in these guidelines are relevant to the product at the time of application for marketing approval. Some relevant information is provided with respect to products in development in these guidelines; otherwise, the respective national regulatory authority should be consulted prior to clinical development on a case-by-case basis (2, 3).

The control and nonclinical testing of each vaccine should be considered individually and any special features should be taken into account. Furthermore, the application of these guidelines to a particular vaccine should reflect its intended clinical use. Thus, different criteria will apply to a vaccine that is to be used prophylactically in healthy children universally, than to one that is to be used therapeutically for treating a life-threatening condition.

3. Definitions

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

Bulk purified plasmid (drug substance). The purified plasmid before final formulation. It is obtained from one or more bulk harvests, and is kept in one or more containers designated as a single homogeneous production lot and used in the preparation of the final dosage form (drug product).

Final formulated vaccine (drug product). The finished formulated vaccine product. It may be freeze-dried and/or contain excipients and/or adjuvants.

Master cell bank (MCB). A homogeneous suspension of bacterial cells, already transformed by the plasmid containing the desired gene, dispensed in aliquots into individual containers for storage. All containers are treated identically during storage and, once removed from it, are not returned to the cell bank.

Plasmid. A circular, extrachromosomal bacterial DNA element which undergoes autonomous replication in bacterial cells. It usually carries a

few genes, some of which confer resistance to various antibiotics; such resistance is often used to discriminate between organisms that contain the plasmid and those that do not.

Working cell bank (WCB). A homogeneous suspension of bacterial cells derived from a single vial of the master cell bank dispensed in aliquots into individual containers for storage. All containers are treated identically and, once removed from storage, are not returned to the cell bank. Typically, a single or a defined number of aliquots is used to manufacture a batch of vaccine. In some cases, a working cell bank may not be established and vaccine manufacture may begin from an aliquot of the master cell bank.

4. **General manufacturing considerations**

Plasmid DNA vaccines are considered to be similar to bacterial and viral vaccines produced by traditional methods, where adequate control of the starting materials and manufacturing process is just as important as that of the product. The guidelines therefore place considerable emphasis on “in-process” controls for assuring the safety and effectiveness of the vaccine as well as on comprehensive characterization of the vaccine itself.

The general manufacturing requirements contained in good manufacturing practices (GMP) for pharmaceutical (4) and biological (5) products will apply to plasmid DNA vaccines. DNA vaccines for use in clinical trials should also be prepared under GMP conditions. Appropriate attention therefore needs to be given to the quality of all reagents used in production, including the components of fermentation media. Many of the general requirements for the quality control of biological products, such as tests for potency, endotoxin, stability and sterility, also apply to DNA vaccines.

It is recognized that the level of detail required by a regulatory agency increases as product development proceeds. During the initial phases of clinical development, the information contained in a clinical trial application should be adequate to allow an assessment of the safety risks from the manufacturing process. This would include, for example, testing of the cell banks for identity, identification and specifications for all materials used in the process, assessment of risks from biologically-sourced materials, a brief description of the process and tests, results of testing of the clinical trial material and preliminary stability of the drug product.

For late-stage clinical trials the level of detail would increase to include preliminary evidence of consistency of manufacture and validation.

Changes made to the product composition (e.g. addition of adjuvant or preservatives) or manufacture (process, site or scale) during the development of clinical and postapproval manufacturing lots may have a significant

impact on safety and/or efficacy. Any change in the production of a DNA vaccine places a responsibility on the manufacturer to show that the product is equivalent to that used in preclinical studies or earlier stage clinical trials. Such changes should be evaluated on a case-by-case basis to determine what supporting data should be provided to show comparability of the modified version with the previous one.

5. **Manufacture and control of bulk purified plasmid (drug substance)**

5.1 **General information**

A brief overview of the development and manufacture of the product including a justification for the selection of the gene(s) of interest, the rationale for the development of the product and the proposed route of administration should be provided.

5.2 **Manufacture**

5.2.1 ***Description of manufacturing process and process controls***

Information should be provided on the manufacturing process, which typically starts with a vial of the cell bank, and includes fermentation, harvest, purification, filling into bulk containers and storage.

A flow chart should be provided illustrating the manufacturing steps from the cell bank up to the drug substance. The chart should include all steps (i.e. unit operations), identification of materials, major equipment and in-process controls.

A description of each process step in the flow chart should be provided and each step justified. Information should be included on, for example, scale; culture media, buffers and other additives; major equipment; and process controls, including in-process tests and operational parameters with acceptance criteria. Information on procedures used to transfer material between steps, equipment, areas, and buildings, as appropriate, and shipping and storage conditions should be provided by the time of application for marketing authorization.

Data on the consistency of fermentation conditions, culture growth and cell and plasmid yield should be presented. Criteria for the rejection of culture lots should be established. The maximum level of cell growth and scale to be permitted during production should be specified, based on information on the stability of the host-cell/plasmid system up to and beyond the level of fermentation used in production by the time of application for marketing authorization.

The characteristics of bacterial cells/plasmids at the end of fermentation should be investigated. This investigation should include, as a minimum, plasmid copy number and restriction enzyme mapping.

For materials such as filter membranes and chromatography resins, information on conditions of use should be provided. Filter membranes and chromatography resins should be dedicated to a single product. In the event that a column or filter is re-used for a single product, conditions of re-use should be provided.

The methods used for harvesting, extraction and purification should be described in detail and justified. The process should be designed to remove process- and host-related contaminants, such as endotoxin, host RNA, chromosomal DNA and any other materials considered undesirable in the final product.

5.2.2 *Control of materials*

The materials used in the manufacture of the drug substance (e.g. raw materials, starting materials, solvents, reagents and catalysts) should be listed and information given on where each material is used in the process. Information on the quality and control of these materials should be provided. Reference to internationally accepted pharmacopoeias or details on the specifications should be provided.

5.2.2.1 *Control of source and starting materials of biological or animal origin*

Information regarding the source, manufacture and characterization of all biologically-sourced materials or materials that may have used biological materials during manufacture should be provided. Summaries of the viral safety information should be provided including appropriate certification where applicable.

5.2.2.2 *Source, history and generation of the host cell*

Information should be provided on the bacterial host cell including its source, phenotype and genotype.

The complete nucleotide sequence of the plasmid DNA should be provided. In addition, the identity, source, isolation and sequence of the gene encoding the antigen(s); a description of the steps involved in the construction of the entire plasmid; a detailed functional map of the plasmid; information on the source and function of component parts of the plasmid known to have biological activities, such as origins of replication, viral/eukaryotic promoters and other expression signals and genes encoding selection markers, should be provided. A clear rationale should be provided for the use of specific regions of DNA, such as the promoter or a gene encoding a selection marker and special attention should be given to the nature of a selection marker.

Any modifications to the original native sequence(s) of the antigen should be described and explained. The location of mammalian promoters in relation to antibiotic resistance genes and the use of novel promoters or inducers should be carefully considered. Certain sequences with properties of mobile elements, such as insertion sequences or retroviral-like long terminal repeats (LTRs), should be avoided. Oncogenes are not recommended unless justified. It is also recommended that genes encoding enzymatic activity or a biological function be either inactivated by genetic manipulation to remove any undesirable activity, or justified. Further, although the relevance at this stage may not be understood, as part of characterization, a DNA sequence homology check of the plasmid with the international databases (e.g. the National Center for Biotechnology Information, National Institute for Health, USA, and/or other international nucleotide databases) should be performed to investigate the presence of unintended sequences of biological significance such as those encoding cellular growth functions or alternative and unanticipated reading frames.

The identity of the plasmid after transfection into the bacterial cell to be used for production should be confirmed in addition to the phenotype of the cell. Representative restriction enzyme maps may be useful. Rearrangements of the plasmid within the host bacterial cell are not acceptable.

5.2.2.3 *Cell banking system, characterization and testing*

The production of a plasmid DNA vaccine should be based on a cell bank system involving an MCB and preferably a WCB. For early stage clinical trials it may be appropriate to use the MCB although sponsors are encouraged to prepare a WCB for later clinical studies.

A well-characterized bacterial cell containing the plasmid should be cloned and used to establish the MCB. The preparation of the MCB and WCB should be conducted according to GMP with appropriate precautions taken to prevent contamination. Information should be provided on the origin, form and storage conditions. Evidence for the viability of the MCB and WCB under storage and recovery conditions should also be provided by the time of application for marketing authorization. New WCBs should be fully characterized and meet established acceptance criteria. Specific phenotypic features that can form a basis for identification of the transformed cell should be described.

The DNA sequence of the entire plasmid should normally be confirmed at the stage of the MCB. Evidence that the MCB and WCB are free from extraneous microbial agents should be provided.

The genetic stability of the plasmid should be confirmed by characterization of the plasmid (copy number, size and sequence) after extended cell growth (end of production) at some stage during development.

5.2.3 ***Process development and in-process control***

The developmental history of the manufacturing process should be provided. Tests and acceptance criteria for critical steps of the manufacturing process should be developed to ensure, and provide feedback on, the control of the process.

Validation of the manufacturing process should demonstrate reproducible and consistent clearance of process and host-related contaminants to levels acceptable for intended use in humans. Data from validation studies on the purification procedures may be required to demonstrate clearance of undesirable contaminants at each purification step and overall.

Process validation is not generally required for a product used in early-stage clinical trials although critical steps such as aseptic processing, sterilization of final product and cleaning validation—particularly when multi-product facilities or contract manufacturing organizations are used for the manufacturing—should be validated or carefully controlled prior to initiation of clinical development.

5.3 **Characterization**

5.3.1 ***Characterization of bulk purified plasmid***

A summary of the characterization of the drug substance should be provided including its identity, strength, biological activity and purity. Rigorous characterization by chemical, physical and biological methods will be essential paying particular attention to the use of a wide range of analytical techniques which are based on different principles.

During development, the sequence of the entire plasmid should be determined. Attention should be paid to possible modification of the DNA because of the possibility that such modifications may influence the biological and immunological properties of the plasmid vaccine.

Potential impurities in the purified product should be described and investigated. These impurities include host cell residues, residual RNA and chromosomal DNA, materials used in the manufacturing process and media components. Data should be provided on the contaminants present in the purified plasmid, with estimates of their maximum acceptable or achievable levels. Denatured plasmid DNA and partial degradation by nucleases are typically observed as part of analytical procedures such as polyacrylamide gel electrophoresis, high performance liquid chromatography and capillary electrophoresis.

5.3.2 ***Consistency of manufacturing***

A number of batches should be characterized as fully as possible to determine consistency. Any differences between one batch and another outside the accepted range for the parameters tested should be noted. The data obtained

from such studies should be used as the basis for the specification. During early clinical development, demonstration of consistency may be limited and occur as manufacturing experience is gained during the clinical development phases. Characterization of consistency of lots is generally done either during phase 3 or, if the phase 3 manufacturing process has not been scaled up for commercial manufacture, after phase 3 and prior to submission of a licence or marketing application.

5.4 Control of bulk purified plasmid

A specification for the drug substance should be established and justified. Descriptions of analytical methods and acceptance limits for the drug substance, including assay validation information should be provided. A summary of the results of testing of all batches produced should be provided.

It is recommended that the specification includes an assessment of the identity, nature and quantity of the plasmid, purity, biological activity, endotoxin content and sterility or bioburden. A justification of the specification should be provided. Early in development the specification may be limited and have wide acceptance criteria.

Not all the tests conducted during product characterization need to be carried out on each batch of vaccine. Some tests are required only to establish the validity or acceptability of a procedure, whereas others might be performed on a limited series of batches to establish consistency of production. Thus, a comprehensive analysis of the initial production batches should be undertaken to establish consistency with regard to identity, purity, potency and stability but thereafter a limited series of tests may be appropriate.

5.4.1 Identity

Each batch should undergo an appropriate selection of the tests used to characterize the purified plasmid in order to confirm its identity. However, the specific tests that adequately characterize any particular plasmid on a lot-to-lot basis, may depend on both the nature of the plasmid and its method of production and purification. Typically, restriction analysis will be the primary approach used to confirm identity; however, *in vitro* or *in vivo* expression of the plasmid accompanied by confirmation of the identity of the expressed antigen should also be considered.

5.4.2 Nature and quantification of plasmid, and biological activity

Quantification of the plasmid is usually by absorbance at 260 nm. The proportion of supercoiled plasmid should be determined and specifications set.

The biological activity of each batch of the drug substance should be determined using a suitable well characterized assay together with an appropriate in-house reference preparation. For biological activity, whenever possible, the antigen should be expressed in vitro by transfection of a suitable cell line and the expressed protein characterized, for example, by immunofluorescence or by Western blot. Where possible, the in vitro assay should be shown to correlate with immunogenic activity or efficacy in an animal model. Alternatively, the plasmid should be shown to possess the relevant immunogenic or biological activity in an animal model.

5.4.3 **Purity**

Limits based on process capability and regulatory guidance should be established for all impurities detected and these should be identified and characterized as appropriate. The degree of contamination with chromosomal DNA, RNA and proteins should be assessed and limits established, and the criteria for rejection should be established and specified. It is important that the techniques used to demonstrate purity be based on as wide a range of physicochemical properties as possible. Where multiproduct facilities or contract manufacturing organizations are used for the manufacturing process, freedom from contamination with other products should be demonstrated.

5.5 **Reference standards or materials**

An in-house reference preparation should be established for use in assay standardization. Information on the reference standards or reference materials used for testing of the drug substance should be provided by the time of application for marketing authorization.

A suitable batch, preferably one that has been clinically evaluated, should be fully characterized in terms of its chemical composition, purity and biological activity, including, where possible, full sequencing, and retained for use as a chemical and biological reference material.

5.6 **Stability**

The stability assessment should be in compliance with the International Conference on Harmonisation (ICH) guideline Q5C “Stability testing of biotechnological/biological products”. The types of studies conducted, protocols used and the results of the studies should be summarized in an appropriate format such as tables, graphs or a narrative document. The summary should include results as well as drawing conclusions with respect to appropriate storage conditions and retest date or shelf-life. Limited stability information would be expected during initial clinical development. Further data on stability to support the expiry date of the drug substance for licence should be based on long-term, real-time stability studies under actual conditions of use.

6. Manufacture and control of final formulated vaccine (drug product)

6.1 Composition

The final composition of the vaccine should be documented.

6.2 Manufacture

A flow chart should be provided that illustrates the manufacturing steps from the bulk purified plasmid to the final formulated vaccine. The chart should include all steps (i.e. unit operations), identification of materials, major items of equipment and in-process controls. In some cases, this may involve simple dilution of the purified bulk; in other cases, a more complex formulation may be envisaged.

A description of each process step depicted in the flow chart should be provided. Information should be included on, for example, scale, buffers and other additives, major equipment, and process controls, including in-process tests and operational parameters with acceptance criteria.

6.3 Control of materials

Details of excipients, adjuvants or any other component of the vaccine in addition to the plasmid constituting the active substance should be provided, including their source, specification, method of conjugation, if appropriate, and final concentration in the vaccine.

6.4 Control of final formulated vaccine

A specification for the drug product should be established and justified. Descriptions of analytical methods and acceptance limits for the drug product, including information on assay validation should be provided. It is recommended that the specification includes an assessment of the identity, nature and quantity of the plasmid, purity, potency, endotoxin content and sterility. A justification of the specification should be provided. Early in development, the specification may be limited with wide acceptance criteria.

A summary of the results of the testing on all batches produced should be provided.

The appropriateness of performing tests on the bulk purified plasmid versus the formulated vaccine should be considered on a case-by-case basis and justified.

Several batches of vaccine, including the final dosage form, should be characterized as fully as possible to determine consistency. Any differences

between one batch and another should be noted. The data obtained from such studies should be used as the basis for the drug product specification.

Not all the tests conducted during product characterization need to be carried out on every batch of vaccine. Some tests are required only to establish the validity or acceptability of a procedure, whereas others might be performed on a limited series of batches to establish consistency of production. Thus, a comprehensive analysis of the initial production batches should be undertaken to establish consistency with regard to identity, purity, potency and stability but thereafter a more limited series of tests may be appropriate.

6.4.1 **Identity**

Each batch of vaccine should be subjected to an appropriate selection of the tests used to characterize the purified plasmid to confirm its identity. The specific tests that adequately characterize any particular plasmid on a lot-to-lot basis, however, may depend on both the nature of the plasmid and the method of its production and purification. Depending on the scope of identification tests, confirmation of the sequence or restriction enzyme mapping and verification of expression following transient transfection, will be necessary.

6.4.2 **Purity**

The purity of each batch of vaccine should be determined and be shown to be within specified limits. The analysis should include sensitive and reliable assays for contaminants of bacterial-cell origin and strict upper limits should be specified for their content in the bulk purified plasmid. Where multiproduct facilities or contract manufacturing organizations are used for the manufacturing process, freedom from contamination with other products should be demonstrated.

6.4.3 **Potency**

The potency of each batch of the vaccine should be determined using a suitable well-characterized assay together with an appropriate in-house reference preparation. A potency assay should be established that can be correlated to functional activity. This may take the form of a quantitative in vitro expression system or may require titration in a defined animal model to determine the minimum quantity of vaccine required to induce an appropriate immune response. The potency of the final vaccine formulation should be established unless otherwise justified and should be correlated with vaccine efficacy.

6.4.4 **Sterility**

Each batch of final product should be tested for sterility.

6.4.5 **Other tests**

The final product specification should include tests for endotoxin, visual appearance and pH. Other tests, such as the residual moisture, if the product is lyophilized, may be required to confirm the physical characteristics of the product as well as the formulation.

6.4.6 **Multi-component vaccines**

Additional factors must be considered when more than one plasmid forms the final formulated vaccine form. Plasmids in multi-component vaccines may encode additional antigens or cytokines or other biologically active molecules which enhance the efficacy or affect the safety of the vaccine. For each plasmid, the development overview, the control of production and the characterization of the bulk purified plasmid must be described as above. Careful consideration has to be given to the control of the final formulated vaccine. For example, potency may depend on the combination of plasmids and their interaction and not on any single plasmid component of a multicomponent vaccine.

6.5 **Reference materials**

A suitable batch of the final formulated vaccine, or bulk purified plasmid, preferably one that has been clinically evaluated, should be fully characterized in terms of its chemical composition, purity and biological activity, including, where possible, full sequencing, and retained for use as a chemical and biological reference material. This material should be used as the basis for evaluation of product quality for production batches.

6.6 **Stability**

Adequate stability studies form an essential part of vaccine development. The stability of the final product in the container proposed for use should, therefore, be determined and the results used to set a shelf life under appropriate storage conditions. Real time stability studies should be undertaken for this purpose, but accelerated stability studies at elevated temperatures may provide complementary supporting evidence for the stability of the product and confirm the stability indicating nature of the assays used to determine stability. The stability assessment should comply with ICH guideline Q5C.

7. **Nonclinical safety evaluation**

Although the recommendations set out below should be considered generally applicable, individual products may present particular safety concerns. The nonclinical safety evaluation should be considered on a product-specific basis taking into account the intended clinical use of the product. It is

important to note that when addressing some of the nonclinical and clinical issues a clear understanding of the product characteristics would be required to enable appropriate studies relating to the toxicology and pharmacology of the product to be designed.

7.1 Introduction

With the advent of DNA vaccines in the 1990s, several potential safety issues associated with administering plasmid DNA to humans were discussed: e.g. integration into the host's chromosomes, immunopathological reactions, risks related to expression of cytokines or co-stimulatory molecules, antibodies against the injected DNA and potential biological activity of the expressed antigen. This revision of the WHO guidelines takes into account the accumulated experience of the use of DNA vaccines in both nonclinical and clinical development. Many phase 1 clinical trials in humans have been conducted with prophylactic DNA vaccines and at least one phase 2 clinical trial with a therapeutic vaccine is in progress. Studies using different doses of up to multi-milligrams, different schedules, routes of administration and delivery devices have been performed. In general, plasmid DNA appears to be safe and well-tolerated, i.e. no serious adverse reactions or major concerns related to the monitored parameters have been noted. At present, prime–boost strategies with other types of vectors, e.g. plasmid DNA/adenovirus and multiple immunization strategies are being investigated to improve immunogenicity. Many animal studies have been performed both with laboratory animals and with veterinary target animal species. It may be useful for sponsors to consult the published literature on experience with relevant animal models in the veterinary field.

7.2 General considerations on safety

This guidance is intended to address the issues specifically related to DNA vaccines. It should be read in conjunction with the guidance provided in the WHO guidelines on nonclinical evaluation of vaccines (2) and the nonclinical section (part A) of the WHO guidelines on clinical evaluation of vaccines: regulatory expectations (3).

The general aim of nonclinical safety evaluation is to determine whether new medicinal products have the potential to cause unexpected and/or undesirable effects. The study design should be based on the intended clinical use and focus on tests that are relevant for a plasmid DNA product. Classical safety or toxicological testing, as recommended for chemical drugs, will have limited application to a plasmid DNA product. As for other biotechnology products, studies with animal models for safety assessment consider two issues: 1) selection of the animal species and physiological state; and 2) the manner of delivery, including the dose, the route of administration and the treatment

regimen (frequency and duration). The nonclinical safety evaluation should provide information about a safe starting dose for the initial human trials. Using animal models to gain knowledge about the response to be expected in humans, always carries the risk that the animal species used in the model does not predict the human response or shows a non-relevant species-specific response. Relevant animal models should be used where possible, i.e. species/models whose immunogenic or biological response to the delivery system and expressed gene product would be expected to be similar to the response in humans.

The risk–benefit evaluation of a product is related to the actual product and its intended use. For example, a prophylactic DNA vaccine for use in healthy children will have a risk–benefit ratio different to that of a therapeutic vaccine against cancer for which there is no other available treatment. Thus, for these and other reasons, it is likely that a flexible approach will be necessary for the nonclinical safety evaluation of DNA vaccines.

If modifications to the manufacturing process or the DNA product are made during the development programme, the potential impact on the product should be considered. Modifications of the genetic sequence, the use of alternative promoter or enhancer sequences, or other changes to the product, may require additional nonclinical safety evaluation. The scientific rationale for the approach taken should be provided.

Although safety testing will undoubtedly be required, the range of tests that need to be carried out should be decided on a case-by-case basis, preferably in consultation with the national control authority. A suitable range of biological, molecular, biochemical, immunological, toxicological and histopathological investigative techniques should be used in the assessment of a DNA vaccine's effect, over an appropriate range of doses during both acute and repeated exposure. Where there is experience with nonclinical and clinical use of a particular delivery vector by a particular route of administration, it may be possible to use information from previous studies or literature in place of further experimental work.

Several potential safety issues associated with administering plasmid DNA into humans are discussed below:

- The injected DNA taken up by cells of the host may integrate into the host's chromosomes and cause an insertional mutagenic event.
- The long-term expression of a foreign antigen may result in an undesired immunopathological reaction.
- The use of genes encoding cytokines or co-stimulatory molecules may pose additional risks.
- Antibodies may be formed against the injected DNA itself and these may contribute towards undesired autoimmune reactions.

- The expressed antigen may itself have biological activity.
- Expression of other gene sequences in mammalian or bacterial cells may pose a risk.

DNA insertion

It is known that DNA taken up by mammalian cells in culture can integrate into the cellular genetic material and be faithfully maintained during replication. This is the basis of the production of some recombinant therapeutic proteins. Theoretically, the introduction of extraneous DNA into a susceptible cell type in vivo could cause a transformation event leading to the formation of tumour cells by the insertion of an active oncogene, by insertional activation of a host-cell proto-oncogene or by insertional deactivation of a suppressor gene. DNA insertion can occur in one of three ways: by random integration of the plasmid into the genome, by homologous recombination between homologous sequences carried by the plasmid and the genome or by a direct integrating mechanism such as is employed by retroviruses. The most likely means in the present context would be random integration.

After injection of naked DNA into an animal, only a small proportion of the DNA molecules enter cells and of those only a fraction are likely to enter the nucleus. The probability of any DNA molecule integrating into a chromosome is very low. When consideration is given to the probability of insertional mutation occurring at a growth regulatory gene and to the multi-step process of oncogenesis, the risk of a tumorigenic event becomes exceedingly low.

Several investigators have assessed the ability of particular plasmids to integrate in vivo based on the association of plasmid DNA with genomic DNA after gel purification (6, 7). In most cases negative results have been obtained at a sensitivity in the region of one potential plasmid integration event per microgram of host-cell DNA (corresponding to approximately 150 000 diploid cells). However, alternative formulations or administration devices such as co-inoculation of a plasmid encoding a growth promoting factor, or electrostimulation, can lead to an increased potential for integration of plasmid DNA, and an investigation of the potential integration of the plasmid DNA in vivo into the host's chromosomes should form part of the nonclinical safety testing of a DNA vaccine. Integration studies may not be necessary for a plasmid DNA vaccine if prior information on a similar plasmid, with the same mode of administration already exists. There would be a need to reassess integration if there was a significant change in the method of delivery, especially any change potentially involving an increase in the capacity of plasmid DNA to enter the nucleus.

Immunopathological reaction

Clinical studies to date have shown that plasmid DNA is well tolerated. Immunopathological reactions such as general immunosuppression and

inflammation have not been reported. Advances in understanding the mechanism of the immune response to an antigen which is expressed from injected DNA have been made although much remains unclear. Knowledge of the duration of expression of an antigen from injected DNA is limited although some reports suggest that expression could continue for many months, which means that the possibility of tolerance may remain a concern. In nonclinical investigations to date, tolerance has not been observed in adult animals and humans and the initial concern may have been overstated. Tolerance can be induced in neonatal mice; this may be because the mouse immune system at birth is immature and if development of tolerance is a concern for a specific product, a more relevant animal model is desirable.

Risks of genes encoding cytokines or co-stimulatory molecules

The co-administration of genes encoding regulatory cytokines or other immunostimulatory molecules has been used to improve the immune response to a DNA vaccine. However, such molecular adjuvants may have additional unintended consequences such as the possibility of stimulating one arm of the immune response at the expense of the other or, in theory, leading to immunopathological reactions, e.g. immunosuppression, chronic inflammation or autoimmunity. Considerable data on the safety (and usefulness) of this approach has accrued especially on their use in clinical trials of gene therapy and therapeutic studies with recombinant protein in humans; however, this approach should be followed carefully. Studies should continue to address the possibility that some cytokines may produce local or systemic toxic effects. The persistence of a cytokine expressing plasmid should be monitored. It cannot be ruled out that studies in animals responsive to the encoded human cytokine or models using the analogous animal genes may be useful to clarify safety issues.

Autoimmune reactions

Bacterial DNA can promote the production of IgG anti-DNA auto-antibodies generally associated with the development of auto-immune glomerulonephritis in diseases such as systemic lupus erythematosus and this initially raised the theoretical concerns that DNA vaccination using bacterial plasmid DNA might induce such disease. Consistent with such a possibility, sensitive enzyme-linked immunosorbent assay analysis of serum samples from humans and animal models have shown that repeated DNA vaccination can stimulate a ≤ 5 -fold increase in anti-DNA auto-antibody levels. Such an increase may not be detected by less sensitive clinical antinuclear antibody screening and the levels observed are well below that associated with the development of autoimmune disease. Although the possibility of anti-DNA antibody production should be considered in the development of plasmid DNA vaccines, analysis in the nonclinical programme is not generally warranted. Improvements in the efficiency of

DNA delivery and/or increases in vaccine dose and frequency may change the need for analysis of anti-DNA antibodies.

Unwanted biological activity

Consideration must be given to the possibility that the in vivo synthesized antigen may exhibit unwanted biological activity. If necessary, appropriate steps must be taken, e.g. by deletion mutagenesis, to eliminate this activity while retaining the desired immune response.

Expression of other gene sequences

If other gene constructs are included in the plasmid, such as antibiotic resistance genes for manufacturing reasons, then the possibility of expression of such gene sequences in mammalian cells or in microorganisms which are potentially pathogenic, and the possible clinical consequences of such expression, should be considered.

It is encouraging that data acquired to date demonstrate the safety of plasmid DNA. The data set is expanding, but the above issues must continue to be addressed, especially as measures are being sought to increase the efficacy of DNA vaccines.

7.3 Considerations on the nonclinical safety programme

In designing the nonclinical safety programme for a DNA vaccine product, the WHO guidelines on nonclinical evaluation of vaccines should be consulted in addition to the guidance provided here. Sponsors are also advised to perform a literature search for relevant published literature which should be used as part of safety evaluation and for justification of their safety programme.

Every product should be evaluated on a case-by-case basis. As a general rule, nonclinical safety assessment should be performed on every novel vaccine or vaccine/adjuvant formulation.

The following parameters should be considered and incorporated into the design of the nonclinical study: the nature of the vaccine, administration route (e.g. intranasal, intravenous, intramuscular or oral), formulation (e.g. liposome encapsulation, poloxamers) and any technique used to improve the uptake of the plasmid (e.g. electroporation). Data from a similar construct may be used to support the nonclinical safety assessment, but will require careful consideration.

When the drug product consists of more than one individual plasmid, testing of the drug product (formulated vaccine) is preferred unless there is reason to suspect one particular plasmid may present a significantly higher risk. The data from a drug product with several plasmids may be applicable for a drug product that contains a subset of the plasmids, unless there is interference between plasmids.

7.4 Toxicity studies

Studies should follow good laboratory practice (GLP) regulations and should test the final formulation intended for clinical use. However, certain assays might be conducted following the principles of GLP when newly developed methods have not been fully established.

The toxicity study may be combined with an assessment of local tolerance, immunogenicity and biodistribution evaluations. When possible, the dose, route and schedule should follow that intended for clinical use and the number of doses should be equal to or exceed that intended in the clinic. The dose interval can be shortened to 2–4 weeks or to another appropriate interval related to the intended dose regimen, although the kinetics of the immune response and potential toxic effect of the gene product should be considered. Relevant biodistribution parameters may also be investigated to allow any findings to be correlated with presence or expression of the gene product.

The animal model should be relevant and the product should be immunogenic in the chosen species. It is recommended that the study include an appropriate number of different doses and a vehicle or other appropriate control group. Toxicity assessments should be performed after both an acute and a recovery (follow-up) period, e.g. at 2–3 days and 14–21 days after the last administration. In general, studies in non-human primates are not required before proceeding to human trials. However, if the expected toxicity is species-specific, primates or transgene mouse models may be more predictive of clinical toxicity. Where possible the same lot of material should be used in the nonclinical safety evaluation and the initial clinical study.

Assessments should include daily clinical observations and injection site reactions, food consumption and body weight. Laboratory assessments should include clinical chemistry and haematology. Postmortem investigations should include macroscopic and microscopic assessments in an appropriate range of tissues, e.g. the injection site, spleen, liver, kidney, intestines, brain, bone marrow, ovaries/testes, lungs, lymph nodes, heart and adrenals. Findings should be assessed in relation to the pattern and severity of the effects and the relevance in relation to the intended product.

The studies should address product-specific concerns including the need for auto-antibody testing. In general, testing for anti-DNA-antibodies is no longer required. Local inflammatory response (e.g. myositis), organ-specific autoimmunity, immunopathology and other relevant parameters may need to be included. In particular where the encoded antigen is a self-antigen, or may show self-antigen mimicry, a wider range of studies, including auto-antibodies, may be necessary.

7.5 Immunogenicity studies

The purposes of the immunogenicity analysis are to define an appropriate dose, route, schedule and formulation of the vaccine for clinical trials and to provide justification (i.e. the benefit side of the risk–benefit equation) and rationale for the clinical trial. The results of assays to show immunological activity in an animal model should be presented. These could comprise antigen-specific antibody titres, serum neutralization titres, seroconversion rates, activation of cytokine-secreting cells and/or measures of cell-mediated immune responses. The studies should optimally be designed to give information about the duration of the immune response and may be combined with challenge or protection studies. If the DNA vaccine is intended to express, for example, a human cytokine, considerations should be given to the species-specificity, i.e. the animal species should be responsive to the cytokine, if possible, or a model using the corresponding animal cytokine may be used.

7.6 Biodistribution, persistence and integration

After inoculation of plasmid DNA into an experimental animal system, assays to assess the distribution, duration and potential integration of the plasmid should be performed, together with an assessment of the germ line, unless otherwise justified.

Biodistribution and persistence studies are required, unless substantial experience has already been gained with an almost identical or similar product. Biodistribution and persistence should be investigated using, for example, sensitive nucleic acid detection techniques, and the justification for the chosen assays should be stated. The assay limit of sensitivity, its specificity, and the potential for tissue- or preparation-specific inhibitors should be established during the assay validation. Among the nucleic acid detection techniques, quantitative polymerase chain reaction (PCR)-based assays have been the most commonly used and most reliable means of assessing plasmid levels in biodistribution and integration studies. The amount of plasmid in the relevant tissues should be quantified and the persistence of plasmid at each site over time should be monitored at an appropriate number of time-points, both early (e.g. 1–7 days) and late (e.g. 2–3 months). The duration and sites of expression of the encoded proteins over time should be investigated. If the encoded protein product is expected to persist for a considerable length of time, the impact of this should be addressed.

Size fractionation/gel-purification assays, where plasmid co-migrates with genomic DNA, may be used as a parameter for potentially integrated plasmid DNA (6, 7). The sensitivity and specificity of the chosen assay should be well-documented.

Published studies on biodistribution of DNA vaccines indicate that intramuscular, subcutaneous, intradermal or particle-mediated delivery does not result in long-term persistence of plasmid at ectopic sites. Some studies have reported ≤ 30 copies of plasmid per 100 000 host cells persist at the site of injection, after 60 days (8–10), whereas other studies have shown that the plasmid can persist at the injection site at levels greater than 500 copies per 100 000 cells 6 months post-administration (7). If ≤ 30 copies of plasmid DNA per 100 000 host cells persist after 60 days, then further integration assessment may not be necessary. As more experience is gained, this issue will need to be addressed again.

Depending on the potential for integration and the proposed clinical indication, further studies may be required to investigate directly any actual integration, e.g. by specially designed PCR-primers (11), or the potential for tumour formation or disruption of normal gene expression.

It may also be necessary to investigate the distribution and clearance of the material used for delivery of the DNA, e.g. complexing material, if sufficient information is not already available in the published literature. The studies should identify sites of uptake after in vivo delivery.

7.7 Genotoxicity

The standard battery of genotoxicity and conventional carcinogenicity studies is not applicable to DNA vaccines. However, genotoxicity studies may be required to address a concern about a specific impurity or novel chemical component, e.g. a complexing material that has not been tested previously.

7.8 Developmental and reproductive toxicity

Integration into reproductive tissue may result in germline alteration. The possibility of distribution to, integration or expression in germline cells must be investigated unless otherwise justified, e.g. the clinical indication or patient population indicates that such studies are not warranted. Confirmation of absence of germline alterations may be gained from investigating the presence and persistence of the plasmid DNA in gonadal tissue from both male and female animals, using e.g. PCR technology. Persistence of plasmids in gonadal tissue over time require further investigation, e.g. of ova and sperm cells and considerations of potential effects on fertility and general reproductive function. In addition, embryo-fetal and perinatal toxicity studies may be required if women of childbearing potential are to be exposed to the product, depending on intended clinical use and population. Such studies may not be required prior to clinical studies in populations with life-threatening diseases, provided appropriate measures are taken to minimize risks. Before a DNA vaccine is used in children or newborns, it

should be tested for safety and immunogenicity in adults, and appropriate nonclinical models, e.g. with juvenile animals, should be considered for the study of toxicity and induction of immunological tolerance.

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