

Annex 1

Guidelines for the production and quality control of synthetic peptide vaccines

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1. Scope

These guidelines are intended to provide a scientifically sound basis for the development, production and control of peptide vaccines for use in humans and to ensure their consistent safety and efficacy. In

addition to providing background information on the development of peptide vaccines, the guidelines cover the following main areas:

- control of starting materials, including background data on the synthesis of the peptide of interest;
- control of the manufacturing process;
- control of the final product.

The guidelines should be read in conjunction with the general manufacturing requirements contained in Good Manufacturing Practices for Pharmaceutical (1) and Biological (2) Products. Guidelines for Good Clinical Practice for Trials on Pharmaceutical Products (3) should also be applied during all stages of the development of synthetic peptide vaccines. Many of the general requirements for the quality control of biological products, such as tests for potency, pyrogenicity, stability and sterility, also apply to synthetic peptide vaccines. The development of synthetic peptide vaccines is at an early stage; no vaccines of this type have yet been licensed, although much research and development is under way and several experimental products are under evaluation. Thus, a flexible approach to the control of these vaccines must be adopted, so that requirements can be modified as experience is gained in their production and use. It is hoped that these guidelines, representing an international consensus view, will be of benefit to those involved in the design and evaluation of peptide vaccines.

Individual countries may wish to use this document to develop their own national guidelines for synthetic peptide vaccines for human use. The guidelines may also, to a considerable extent, be relevant to synthetic peptide vaccines for veterinary use.

These guidelines are intended to facilitate the process of submission and evaluation of a peptide vaccine for licensing. Experience has shown that the process of obtaining a licence is much easier if the considerations set out below are kept in mind from the earliest stages of development.

2. Introduction

Traditional vaccines have been prepared either by using an attenuated version of a pathogen, such as for yellow fever, poliomyelitis and measles vaccines or BCG, or by preparing and inactivating a disease-causing organism (such as for pertussis, influenza and typhoid vaccines) or a suitable part of it, for instance, a toxin, as in the case of diphtheria and tetanus vaccines, and administering it in quantities

sufficient to induce immunity. These approaches have led to many effective vaccines. However, difficulties often remain in the manufacture of vaccines, in maintaining their efficacy and in ensuring their safety on a lot-by-lot basis.

For attenuated vaccines, examples of common difficulties are:

- developing stable attenuated strains of the pathogen;
- the reversion to virulence of the attenuated strains;
- controlling strain properties;
- the presence of adventitious agents;
- the necessity for a cold chain.

For inactivated vaccines, examples of common difficulties are:

- the necessity of achieving total inactivation;
- avoiding reactogenicity while retaining protective immunogenicity.

Common difficulties for both types of vaccine are:

- the culture of pathogenic organisms in the amounts necessary for production;
- the possibility of inducing an inappropriate immune response (cross-reactivity with host antigens, for instance).

In addition to the traditional use of vaccines for protection against infection and toxins, there is great interest in the potential of immunotherapeutic techniques in such areas as stimulating immunity to cancer cells, down-regulating the effects of polypeptide hormones, and controlling inappropriate immune responses (such as anaphylaxis).

2.1 The potential of peptides as vaccines

An alternative approach to immunization would be to identify on immunogens the peptide epitopes that induce the requisite response and to use synthetic versions of these peptides in the production of vaccines. Unlike traditional vaccines, such vaccines, because they are totally synthetic, would not carry the risk of reversion or of incomplete inactivation, and, in principle, epitopes could be selected to avoid components that give rise to unwanted side-effects. In addition, the use of synthetic peptides makes available antigens that may be difficult to prepare in quantity from a natural source, such as parasite antigens, offers the prospect of eliciting immunity to antigens that are not normally recognized, for instance, “self” antigens such as tumour-specific antigens in cancers, and makes it possible to elicit a response to epitopes that remain cryptic during natural infection.

2.2 Peptides and the immune system

For a peptide (or other antigen) to stimulate an effective immune response, it must be efficiently processed and presented to T cells. T cells recognize the antigen or peptide by the different MHC (major histocompatibility complex) molecules on the surface of the antigen-presenting cell, and this is critical in determining the nature of the immune response generated. The type of MHC-antigen complex that results is dependent on the source of the antigen or peptide. Proteins derived from inside a host cell (e.g. viral proteins) are usually processed via the MHC class-I pathway and elicit predominantly cytotoxic T-cell responses. Exogenous antigens (e.g. proteins or peptides excreted by bacteria) are taken up by specialist antigen-presenting cells and normally processed via the MHC class-II pathway. This elicits CD4 T-helper (Th) responses that in turn can lead to either a predominantly humoral response resulting in antibody formation or a predominantly cell-mediated response characterized by the activation of macrophages, the expansion of the antigen-reactive T-cell pool and the production of cytokines.

An effective peptide vaccine must be targeted to the appropriate processing pathway to elicit the desired response in the host. This may be achieved by a variety of strategies based on an understanding of the processing of the antigen *in vivo* and known antigenic epitopes. Peptide vaccines may be linked to macromolecular carriers (such as tetanus toxoid), used in combination with lipid micelles or be expressed with specific peptide modifications (such as linking to ubiquitin) that target different processing pathways.

2.3 Potential limitations of the peptide approach

The approach outlined is not without practical and theoretical difficulties. In practice, the choice of epitope to synthesize is restricted to linear epitopes. A major limitation of this approach is that the conformational B-cell epitopes which are involved in the neutralization of, for instance, some viruses and toxins are difficult to mimic by a simple synthetic analogue. One approach that attempts to address this problem is the synthesis of template-assembled synthetic proteins (TASPs), in which assembly of peptides on a suitable “backbone” ensures the formation of the desired three-dimensional structural elements. Another possible problem is that, if immunization is based on a few epitopes, the frequency of antigenic variants that do not cross-react may be relatively high, and the vaccine may serve only to select for a sub-population of the pathogen that evades immune surveillance. There is some evidence for this in vaccinees receiving recombinant hepatitis B surface antigen (HBsAg). Because of the

limited repertoire of Th epitopes available in a vaccine based on a limited number of peptides, the immune response may be MHC restricted, which raises the possibility of a limited and selective response in the target population. However, it may be possible to cover MHC variation in the target population by the use of multiple or promiscuous T epitopes, and to include multiple B-cell epitopes to address antigenic diversity. Antibodies recognizing a peptide immunogen may be readily obtained, but, as with other approaches, this response may be neither sufficient nor appropriate for protection.

Antibodies elicited in response to peptides may have unexpected cross-reactivity to normal tissues, possibly giving rise to an autoimmune reaction. This is a major concern in some areas of immunotherapeutic vaccination, but may arise in more conventional types of vaccine, for instance where an infectious agent has adopted the strategy of host mimicry to evade an immune response.

3. **Delivery to the immune system**

Small peptides by themselves are poor immunogens, with short physiological lifetimes, and for an efficient immune response it is necessary to take special steps to enhance their stability and delivery to the immune system. These approaches include: the construction of retro-inverso peptides (4), where physiological persistence is enhanced by the construction of an analogue of an epitope by synthesizing the inverse sequence using D-amino acids; polymerization of a peptide (for instance by disulfide cross-linking (5, 6)); construction of dendrimers, such as multiple antigen peptides (MAPs) (7) and TASPs (8); and conjugation to a suitable carrier. One major difficulty with peptides conjugated to a macromolecular carrier is that the immune response can be dominated by immune response to the carrier or, even worse, suppressed by the carrier (usually a protein). Peptides may also be synthesized with lipophilic or glycosidic functional groups that improve delivery and targeting to antigen-presenting cells and may enhance incorporation into vehicles or adjuvants (9, 10). All of these approaches may be used singly or in combination. However, it is clear that one critical factor in the type of immune response elicited is likely to be the choice of adjuvant.

Adjuvants are materials administered at the same time as an antigen to enhance the immune response. Different adjuvant components may serve different purposes (10, 11). Some may act simply as vehicles for the immunogen, allowing it to be more efficiently made available to the antigen-presenting cells, while others may act to

target particular types of antigen-presenting cells through interaction with specific cell receptors, to enhance pinocytosis by the antigen-presenting cells, or as immunomodulators, which may stimulate either a general up-regulation of the immune system or direct the development of immunity into either the cellular or the humoral branch. Until very recently, almost the only kinds of adjuvant in use in vaccines for humans have been the mineral gels aluminium hydroxide and calcium or aluminium phosphate, all of which are excellent at directing a humoral response, but are poor stimulators of cellular immunity. However, other adjuvants are now appearing as components of vaccines that have been licensed or are in advanced stages of clinical trial. Many candidate adjuvants consist of mixtures of components with different activities. Antigens may be incorporated into particulate or liposomal preparations or into emulsions which may contain other components to stabilize and modify the activity, as with immune-stimulating complexes (ISCOMs), which contain cholesterol, phospholipids and saponins. Other proposed adjuvants are based on bacterial cell-wall components (muramyl dipeptide), non-ionic block copolymers, saponins, carbohydrate polymers and polysaccharides, cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-1, -2, -4 and -12 or bacterial (lipid A, proteosomes) or viral components. See reference 12 for a compendium of adjuvants and reference 10 for a tentative classification of their modes of action.

Many new adjuvants raise issues of toxicity and safety that will need to be addressed on an individual basis and in conjunction with the antigens with which they are to be used.

4. **Peptides and their synthesis**

The synthesis of even a moderate-sized peptide involves a very extended sequence of reactions — several for each amino acid added. Before the α -amino group of one amino acid can be combined with the carboxyl group of another to form a peptide bond, one of the two groups must be “activated” by conversion to a highly reactive derivative. The choice invariably falls on the carboxyl group, and synthesis thus proceeds from the C- to the N-terminus. If the carboxyl group of amino acid A is to be combined with the α -amino group of amino acid B to produce the dipeptide A–B, then the amino function of A and the carboxyl function of B have to be protected by conversion into forms that will allow the formation of only the specific peptide bond between A and B. In addition, amino acid side-chain groups that will react with an activated carboxyl must also be protected. The

side-chain-protecting groups used must be capable of withstanding the conditions used to remove the α -amino-protecting group in order to produce a fresh free amino group for continuation of the peptide elongation. At the end of synthesis, all the protecting groups must be removed to reveal the desired peptide. Peptide synthesis thus relies on a series of reactions in which a fresh peptide bond is created during each cycle. Peptide synthesis is most efficient for the synthesis of peptides of lengths up to 50 residues. From about 50 residues upwards, recombinant techniques, in which a synthetic DNA sequence is expressed in a suitable vehicle, become more attractive, though technical issues (such as the incorporation of non-natural amino acids) may still weigh the choice in one direction or another.

There are two main strategies for synthesis: in the classical solution (fragment-condensation technique) a number of small peptides are synthesized first. These are then purified, deprotected and recombined to form larger peptides, and so on, until the final coupling produces the desired sequence. This technique allows the maximum flexibility in the choice of coupling chemistry and the combination of blocking groups, and also offers the opportunity to characterize and purify intermediate peptides as the synthesis proceeds. On the other hand, large protected peptides are difficult to handle because of problems of solubility, and because reaction products and solvents are difficult to remove. With this approach, each synthesis is an optimized "one-off" design.

In the solid-phase ("Merrifield") method of sequential synthesis, problems of handling are resolved by coupling the C-terminal amino acid to a solid resin bead. The peptide is constructed by stepwise addition of amino acids at the N-terminus. Attachment to a solid support allows reagents to be easily removed at each coupling cycle. At the end of synthesis all the protecting groups are removed (deprotection) and the peptide is cleaved from the resin support in a single- or double-stage reaction. Solid-phase methods are conveniently classified into two main types: tBoc and Fmoc, named after the N-terminal protecting groups that determine the type of chemistry to be used. The former (tBoc) exploits an acid-labile N-terminal and side-chain protecting group, with cleavage and deprotection achieved simultaneously by use of very strong acid (hydrogen fluoride or trifluoromethane sulfonic acid). The Fmoc method employs a base-labile N-terminal protecting group in combination with acid-labile side-chain protecting groups. The side-chain protecting groups can be removed and the peptide simultaneously cleaved from the support under less extreme conditions than with the original tBoc method.

Within this broad framework there are many variations, and the field is continually evolving with the introduction of improved protecting groups, new supports, better cleavage reagents, the exploitation of enzymic methods for peptide-bond formation, and so forth. In the field of vaccine synthesis additional complexity is introduced by techniques in which the peptide is attached to or assembled on a backbone or skeleton designed to provide desirable structural (TASPs) and immunogenic properties (TASPs and MAPs), or where peptides are modified with lipid or carbohydrate groups. Both liquid- and solid-phase methods work well in experienced hands, but solid-phase methods are simpler in conception, have fewer variables, are easily automated and are far more rapid. They are thus well suited to the non-specialist producer of synthetic peptides. They are perhaps not so well adapted to large-scale use, but several small-scale solid-phase syntheses can be carried out in the time taken for one synthesis in solution. In practice, solid-phase methods now heavily predominate, particularly in such areas as epitope mapping, and are likely to provide the method of choice for synthetic peptide vaccines.

5. **Characterization and control**

In the case of an industrial-scale “classical” fragment condensation, the process of synthesis and purification can easily take more than a year. At the end of the synthesis, the product is inevitably contaminated by a wide range of closely related by-products resulting from side-reactions and partial reactions which may occur at each cycle of synthesis. These by-products vary greatly in relative amount, but it is often possible to demonstrate as many as 100 individual species for a large peptide. For a conventional “pharmaceutical” — a peptide hormone, for instance — the product often needs to be extensively purified to reduce the level of impurities. It is often assumed that the purity requirements for peptides for immunological use are much less demanding, but there is evidence that specific minor impurities may significantly interfere with the immunological properties of a peptide preparation (13, 14).

When synthetic peptides first started to replace peptides of natural origin for therapeutic use, control procedures were very much based on those developed for the natural product: definitive tests relied very heavily on bioassays, and though physicochemical tests were included in specifications, these were a comparatively modest part of the whole. It is only recently that the greatly increased resolving power and quantitative abilities conferred by modern analytical methods — notably high-performance liquid chromatography and

mass spectrometry — have allowed the identification, assay and purity testing of pharmaceutical peptides to be based almost entirely on physicochemical techniques. However, full biological characterization retains a critical role in product development. Synthetic pharmaceutical peptides are well established in therapeutic use, with a history going back almost 30 years. Examples of peptides in everyday therapeutic use include oxytocin, vasopressin and analogues, gonadorelin (gonadoliberin) and its analogues, the calcitonins and corticotropin and its analogue tetracosactrin.

Pharmacologically active peptides are administered either alone (usually by injection) or sometimes incorporated into a formulation designed to delay release. None is covalently linked to a complex polymer. In contrast, a peptide antigen selected for vaccine development may be conjugated to a macromolecular carrier or incorporated into a polymeric structure that may be important for its immunogenic properties, but may also lead to undesirable pharmacological properties of either the peptide or its impurities. Evidence of consistency from batch to batch of the peptide is extremely important and should be based on a wide range of physicochemical analytical techniques designed to address complementary properties of the product. These might, as appropriate, include analytical techniques based on the following: distinctions of charge (electrophoretic techniques such as polyacrylamide gel or capillary electrophoresis; cation- or anion-exchange chromatography and isoelectric focusing and chromatofocusing); distinctions of size (size-exclusion chromatography, SDS-polyacrylamide gel electrophoresis — both reducing and non-reducing); distinctions of hydrophobicity (reversed-phase or hydrophobic-interaction chromatography); or distinctions of mass (mass spectrometry based on a range of ionization methods). Also included may be analytical techniques based on determination of composition (amino-acid analysis), sequence (sequential degradation from the N-terminal (Edman degradation) or C-terminal (chemical or enzymic degradation)), mass spectrometry, and peptide mapping following enzyme or chemical (e.g. cyanogen bromide) digestion.

In a similar fashion, it is important to demonstrate the consistency of successive stages of manufacture, as the peptide is incorporated into or combined with other components. The techniques chosen should be appropriate for demonstrating consistency of stoichiometry of combination, and should, as above, be as widely based as possible. In addition to the suggestions mentioned above, additional techniques based on other physicochemical techniques or characteristics (e.g. ultraviolet spectroscopy, circular dichroism, fluorescence spectroscopy, light scattering) may be appropriate, as well as immunologically

based techniques. Information gained from clinical use may also be relevant.

More detailed information relating to the development and use of synthetic peptides as vaccines can be obtained elsewhere (15–29).

6. **Developmental overview**

Because of the range of preparations that can make up a peptide vaccine, it is not possible to give detailed recommendations covering all examples. However, adequate control of the starting materials and manufacturing process is just as important as control of the final product. Considerable emphasis should therefore be put on “in-process” controls, as well as on the comprehensive characterization of the vaccine itself, for ensuring the safety and efficacy of the vaccine. Appropriate attention therefore needs to be given to the quality of all reagents used in production. Although these guidelines set out points which should be considered when developing synthetic peptide vaccines, not all points will be appropriate to all vaccines. Individual vaccines may present particular control problems. The production and quality control of each vaccine must therefore be given careful individual consideration so that any special features can be taken into account. Furthermore, the application of these guidelines to a particular product should reflect its intended clinical use.

A full description of the development of the vaccine should be provided to the national control authority, including the identification of the antigenic determinants, the reasons for choosing these particular determinants, and the rationale behind such aspects as choice of carrier, combination of epitopes, choice of conjugate, form of presentation of the peptides and choice of adjuvant, together with any evidence bearing on these choices.

The different elements of the vaccine should be rigorously characterized, both individually and in combination, to ensure that the final product is safe, effective and of consistently reproducible quality. A wide range of physicochemical techniques designed to address complementary properties of the product should be used. The techniques should provide evidence of correct sequence and purity, together with evidence for the correct and quantitative incorporation of modifications such as glycosylation or lipidation. In addition, a range of immunological techniques should be used to evaluate the antigenic and biological properties of the product and its components, including immunogenicity, antigenicity, specificity and biological potency. Techniques will need to be selected on a case-by-case basis.

Because of the complexity of the possible antigenic constructs and the analytical problems involved in characterizing polymers and conjugates, it is important that a reference preparation of the antigen be established. Such preparations should be representative of, or prepared from, batches of material used for clinical studies. It may be necessary to prepare different reference preparations appropriate to separate stages of manufacture.

7. Control of production

7.1 Peptide synthesis and characterization

The epitopes within each peptide sequence should be described and identified. There should be a description of the source of each epitope and, where there is more than one epitope, the reasons for the sequence, combination, and type of epitope (B, T, etc.) should be specified.

A full description of the synthesis of the peptide and in-process controls should be given. The description should include sources and specifications of starting materials, methodology used, coupling and deprotection conditions, criteria for proceeding to the next step of synthesis, and details of any groups (glycosyl or lipid, for instance) incorporated. If synthesis is performed in solution, a flow chart and specifications of the intermediate peptides should be supplied.

Where a peptide monomer is an intermediate, it should be isolated and characterized. Evidence should be provided to confirm that:

- the principal peptide sequence is the intended structure, i.e. that the correct amino acids have been added in the intended order and that the correct number and type of modifications (e.g. incorporation of lipid or glycosyl moieties) have been carried out;
- major and, where possible, minor impurities in the peptide monomer have been identified and characterized. Techniques used to demonstrate purity should be based on as wide a range of physicochemical properties as possible. It may be necessary to test for materials added during the production or purification processes;
- the peptide is of consistent quality over a number of consecutive batches.

A suitable specification for the monomeric peptide or peptide derivative should be established. This may need to be defined, in part, in terms of a suitably characterized reference preparation (see above).

Limits should be set for peptide purity, for individual major and minor impurities and for total impurities. If full characterization of the peptide monomer is not possible, a detailed explanation should be given. Finally, studies should be carried out to assess the stability of the peptide for proposed periods of storage.

7.2 Conjugates

In addition to recommendations regarding the peptide and other components of the conjugate (see section 7.3), conjugated product should meet the following criteria:

- Proof of conjugation should be established, and the level of conjugation should be consistent and reproducible. Where more than one peptide is conjugated to the same carrier, the proportions of each should be reproducible.
- Side reactions (e.g. cross-linking of carrier) should be minimized, as should the surface concentration of non-productive coupled linker on the carrier and the presence of linker not involved in coupling. Related parameters should be reproducible.
- Evidence should be obtained that conjugation does not alter the antigenic sequences.
- Levels of residual reagents and by-products should be minimized (defined limits should be set).

In addition, the following issues are important:

- A suitable acceptance specification for the conjugated product should be set. A suitable reference preparation may be necessary.
- Potential clinical effects of pre-existing or induced immunity to the carrier protein should be considered.
- Specific epitope suppression should be investigated when a carrier is used for several vaccines or for several different epitopes of one vaccine.

7.3 Carriers

Peptides may be conjugated to or incorporated into a carrier. Care should be taken in choosing a carrier, since an immune response to the carrier may dominate response to the peptide. Any carrier to which pre-existing hypersensitivity in the target population is likely should be avoided, as should carriers that involve the risk of stimulating autoimmunity.

Carriers should have a complete specification and dossier of background information. This information should include characterization, evidence of consistency of production, safety-testing data and other information appropriate to the protein, polysaccharide, polymer, liposome etc., as the case may be. For instance, polymers and polysaccharides should have a reproducible molecular-weight distribution, polysaccharides should have consistent monosaccharide composition, and liposomes a consistent dimension and composition. Components and impurities should be characterized and quantified. For a carrier of biological origin, measures should be undertaken to ensure the absence of infectious agents.

7.4 **Polymerized, cyclized or carrier-synthesized peptides**

Where a peptide is synthesized on a polymeric backbone (such as in MAP synthesis) or allowed to polymerize or cyclize following synthesis (for instance, through oxidation of terminal cysteine residues), either alone or in a copolymerization with other peptides, evidence should be obtained to ensure that the reaction proceeds to a reproducible endpoint. In particular, the following should be ensured:

- The distribution of relative molecular mass of the product is consistent from batch to batch, so that, for a cyclized product, it is shown to be predominantly and consistently the cyclic monomer (for instance) and polymeric forms are shown to be within the limits of specification; for a polymer, that the level of monomer is within specified limits, and the pattern of polymerization is defined (for example, in terms of molecular-mass distribution and heterogeneity (M_z/M_w , M_w/M_n , etc., where “M” stands for relative molecular mass and “Z”, “W” and “N” refer to specific polymers), or in terms of limits set for the proportions of specific polymeric species).
- Where appropriate, the relative proportions of different peptides are consistent.
- The nature of the product(s) is well defined by means of an appropriate range of complementary analytical techniques, which might include techniques exploiting differences in charge, molecular size, hydrophobicity, mass, or spectral or immunological properties (see p. 32).
- Levels of reagents used in polymerization, cyclization or synthesis, together with unwanted by-products, are within the limits that have been set.

Where successive production stages result in polymers of increasing complexity (as, for instance, in MAP synthesis followed by disulfide

dimerization of the product), it may be appropriate to apply the above-mentioned criteria after each production stage.

A suitable acceptance specification for the oligomeric product should be set and a reference preparation established (see pp. 39–40).

7.5 **Adjuvants, vehicles and excipients**

A wide range of different materials may in principle be added to an immunogen in order to improve handling, stability and persistence and to modify the degree and type of immunogenicity. Such materials vary widely in their characteristics and range from small molecules to bacterial-cell components and synthetic polymers. They may include components acting as general immunostimulants, components selectively activating different parts of the immune system, and components interacting directly with the different cells involved in the immune process. Many adjuvants can present questions of toxicity, and, because of the way in which they can modify the immune process, raise safety concerns when combined with an antigen. Traditional adjuvants have been based on mineral gels (aluminium hydroxide, aluminium or calcium phosphate); few others have so far been licensed.

Established adjuvants based on aluminium or calcium salts should be of the requisite pharmacopoeial grade (i.e. free of heavy metal ions and of consistent quality and binding characteristics). Since operations such as autoclaving may alter an adjuvant's binding characteristics, it is important that batches of adjuvant should be handled in the same well defined manner. If aluminium or calcium compounds are used as adjuvants, their concentrations should not exceed the customary limits of 1.25 mg of aluminium and 1.3 mg of calcium per single human dose.

For additives, a full description of all components should be provided, together with a detailed rationale for their inclusion. Each component should have an appropriate analytical specification, preferably on the basis of a drug master file, and there should be a specification for the mixture of components in the final formulation. In addition, there should be a full assessment of the antigenic and toxic properties of the final formulation, with attention given to the possibility that antigenic properties may be adversely modified through the combination of components.

7.6 **Preservatives**

If a preservative is added, the preservative content should be determined and the amount used should be shown not to have any dele-

rious effect on individual vaccine components, nor cause any unexpected adverse reactions in humans. Preservatives should not be added to single-dose preparations.

8. **Formulated final product**

After combination of the antigen with adjuvant and any cofactors, additional characterization should be carried out as indicated below.

8.1 **Interaction with adjuvant**

The degree and manner in which the different vaccine components interact with the adjuvant should be defined, and reproducible behaviour from batch to batch demonstrated. For instance, the degree to which the different species in a polymeric preparation bind to the adjuvant may depend on molecular mass, hydrophobicity or pH, and the preparation may therefore effectively become fractionated during formulation. Alternatively, components of the adjuvant may associate as micelles, and the different vaccine components may be incorporated to different extents. Desorption of vaccine components from the adjuvant may take place over time, and specifications should be set to ensure consistency.

8.2 **Stability**

Adequate stability studies form an essential part of vaccine development. The results are used to set a maximum shelf life under appropriate storage conditions. For each component for which a claim of efficacy is made, real-time stability studies should be undertaken to support the immunogenicity and lack of toxicity of the product up to its expiry date. Accelerated stability studies may provide preliminary supporting evidence for the stability of the product, but cannot replace real-time studies for the purpose of licensing.

Guidance on stability studies can be found in the International Conference on Harmonisation's (ICH) guidelines on stability testing.¹

8.3 **Potency**

The potency assay need not necessarily reflect the functional activity of the vaccine or its mechanism of action in humans. However, a

¹ *Quality of biotechnological products: stability testing of biotechnological biological products*. Geneva, International Conference on Harmonisation, 1995 (available from ICH Secretariat, International Federation of Pharmaceutical Manufacturers Associations, 30 rue de St-Jean, 1211 Geneva 18, Switzerland).

suitable test of vaccine function should be included where appropriate. The main purpose of the potency assay is to demonstrate batch-to-batch consistency by methods which depend in some measure on biological activity. A suitable *in vitro* or *in vivo* assay for immunogenicity or antigenicity should therefore be considered. Such an assay should compare the preparation with a reference material, and the results should be statistically validated. Since the mode of action of vaccines may vary, details of such assays must be determined on a case-by-case basis. However, confidence limits should be calculated for the mean value of any batch in order to take account of inherent variation in the bioassay. Acceptance criteria should be defined on this basis. A suitably formulated reference preparation should be established for this purpose.

9. **Routine control**

Routine testing is that which is carried out on every batch, both of the component products and of the final formulation. For each stage of production, intermediate products should be subject to an acceptance specification, and a suitable range of tests carried out to ensure compliance. Such tests are usually a subset of those carried out for developmental characterization. They should test aspects of the intermediate and final product that are critical to safety, efficacy and quality.

The product specification should also include tests to ensure that the final dosage form complies with the usual safety tests, such as pyrogenicity and sterility, appropriate to a parenteral preparation, together with tests of identity, antigen content and general innocuity.

Consistency of production is essential. Demonstration of consistency of production should include the results of tests on at least three satisfactory, consecutive production batches of a size corresponding to that intended for routine manufacture. The data obtained from such studies may be used as the basis for the final vaccine specification.

The final product specification should also include tests to ensure that the content of any preservatives is within specified limits.

10. **Reference materials**

The studies described in sections 7 and 8 will contribute to a definitive specification for the vaccine.

It may be necessary to establish appropriate reference materials. In addition, a suitable batch of the final formulated vaccine, preferably one that has been clinically evaluated, together with relevant intermediate products, should be fully characterized in terms of its chemical composition, purity and biological activity and retained for use as a chemical and biological reference material. This material should be used as the basis for defining the specifications for production batches.

11. **Preclinical safety evaluation**

The general aim of preclinical safety evaluation is to determine whether new medicinal products have the potential to cause unexpected and undesired effects. Clinical safety or toxicological testing as recommended for chemical drugs may, however, be of only limited relevance for synthetic peptide vaccines. Toxicity testing in animals poses particular problems, such as those due to species specificity, and the safety evaluation of peptide vaccines will have to take a large number of factors into account.

For these reasons, it is likely that a flexible approach will be necessary for the preclinical safety evaluation of synthetic peptide vaccines. Account should be taken of any possibility of adverse immunopathological reactions arising from the use of a synthetic peptide vaccine.

It is important to check that the peptide sequences used possess no significant unwanted pharmacological activity. The peptide monomer should therefore be screened for intrinsic toxic or pharmacological activity. The potentiation of any unwanted pharmacological activity through conjugation or polymerization should also be considered (16). A peptide may well have unanticipated but minor pharmacological effects that could be significantly and harmfully magnified by conjugation or polymerization. The pharmacological activity of both peptide and conjugate should therefore be examined.

Antibodies elicited in response to peptides may have unexpected cross-reactivity with human tissues and elicit an autoimmune reaction. Any undue cross-reactivity of antibodies (induced with the final vaccine formulation) with intrinsic human antigens should be assessed using a panel of human tissues. Conjugation and polymerization can create epitopes that would not be addressed by evaluation of only the peptide monomer.

Although safety testing will be required, the range of tests that need to be carried out should be decided on a case-by-case basis, and in

consultation with the relevant national control authority. For preclinical safety evaluation, a wide range of biological, biochemical, immunological, toxicological and histopathological techniques should be used as appropriate, covering a relevant range of doses and including both acute and chronic exposure.

Authors

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References

1. Good manufacturing practices for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations. Thirty-second*

- report. Geneva, World Health Organization, 1992, Annex 1 (WHO Technical Report Series, No. 823).
2. Good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization. Forty-second report*. Geneva, World Health Organization, 1992, Annex 1 (WHO Technical Report Series, No. 822).
 3. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: *The use of essential drugs. Sixth report of the WHO Expert Committee*. Geneva, World Health Organization, 1995, Annex 3 (WHO Technical Report Series, No. 850).
 4. Benkirane N et al. Mimicry of viral epitopes with retro-inverso peptides of increased stability. In: Brown F, ed. *New approaches to stabilisation of vaccine potency: proceedings of a symposium, WHO headquarters, Geneva, May 29–31, 1995*. Basel, Karger, 1996 (Developments in biological standardization, vol. 87).
 5. Patarroyo ME et al. A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum*. *Nature*, 1988, **332**:158–161.
 6. Calvo M et al. Specific interaction of synthetic peptides derived from *P. falciparum* merozoite: proteins with human red blood cells. *Peptide research*, 1991, **4**:324–333.
 7. Nardelli B, Tam JP. The MAP system: a flexible and unambiguous vaccine design of branched peptides. In: Powell MF, Newman MJ, eds. *Vaccine design: the subunit and adjuvant approach*. New York, Plenum, 1995:803–819.
 8. Tuchscherer G, Mutter M. Templates in protein *de novo* design. *Journal of biotechnology*, 1995, **41**:197–210.
 9. Deres K et al. *In vivo* priming of virus-specific cytotoxic lymphocytes with synthetic lipopeptide vaccine. *Nature*, 1989, **342**:561–564.
 10. Cox JC, Coulter AR. Adjuvants: a classification and review of their mode of action. *Vaccine*, 1997, **15**:248–256.
 11. Allison AC, Byars NE. An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell-mediated immunity. *Journal of immunological methods*, 1986, **95**:157–168.
 12. Vogel FR, Powell MF. A compendium of vaccine adjuvants and excipients. In: Powell MF, Newman MJ, eds. *Vaccine design: the subunit and adjuvant approach*. New York, Plenum, 1995:141–225.
 13. Ede NJ et al. Identification and synthesis of altered peptides modulating T-cell recognition of a synthetic peptide antigen. *Biomedical peptides, proteins and nucleic acids*, 1995, **1**:231–234.
 14. Chen W et al. CTL recognition of an altered peptide associated with asparagine bond rearrangement. *Journal of immunology*, 1996, **157**:1000–1005.
 15. Francis MJ. Peptide vaccines for viral diseases. *Science progress*, 1990, **74**:115–130.

16. Arnon R, Horwitz RJ. Synthetic peptides as vaccines. *Current opinion in immunology*, 1992, 4:449–453.
17. Mills KHG. Recognition of foreign antigen by T cells and their role in immune protection. *Current opinion in immunology*, 1989, 2:804–814.
18. Sesardic D. Synthetic peptide vaccines (editorial). *Journal of medical microbiology*, 1993, 39:241–242.
19. Romero P. Malaria vaccines. *Current opinion in immunology*, 1992, 4:432–441.
20. Del Giudice G. New carriers and adjuvants in the development of vaccines. *Current opinion in immunology*, 1992, 4:343–459.
21. Partidos CD, Obeid OE, Steward MW. Antibody responses to non-immunogenic synthetic peptides induced by co-immunisation with immunogenic peptides. *Immunology*, 1992, 77:262–266.
22. Christodoulides M, McGuinness BT, Heckels J. Immunization with synthetic peptides containing epitopes of the class-I outer-membrane protein of *Neisseria meningitidis*: production of bactericidal antibodies on immunization with a cyclic peptide. *Journal of general microbiology*, 1993, 139:1729–1738.
23. Dintzis RZ. Rational design of conjugate vaccines. *Pediatric research*, 1992, 32:376–385.
24. Melen RH et al. Synthetic peptide vaccines: success at last (comment). *Vaccine*, 1995, 13:885–886.
25. Langeveld JPM et al. First peptide vaccine providing protection against viral infection in the target animal: studies of canine parvovirus in dogs. *Journal of virology*, 1994, 68:4506–4513.
26. Tam JP. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proceedings of the National Academy of Science (USA)*, 1988, 85:5409–5413.
27. Briand JP, Muller S, van Regenmortel MHV. Synthetic peptides as antigens: pitfalls of conjugation methods. *Journal of immunological methods*, 1985, 78:59–69.
28. Kriwaczek VM et al. Superpotency and superaffinity phenomena in the stimulation of steroidogenesis in adrenocortical cells by adrenocorticotropin–tobacco mosaic virus conjugates. *Molecular and cellular biochemistry*, 1981, 40:49–59.
29. Dong P, Brunn C, Ho RJY. Cytokines as vaccine adjuvants: current status and potential applications. In: Powell MF, Newman MJ, eds. *Vaccine design: the subunit and adjuvant approach*. New York, Plenum, 1995:625–643.