administration should correspondent to that intended for use in the clinical trials. When the vaccine is to be administered in human clinical trials using a particular device, the same device should be used in the animal study, where feasible (e.g., measles aerosol vaccine in the monkey model). Potential toxic effects of the product should be evaluated with regard to target organs, dose, route(s) of exposure, duration and frequency of exposure, and potential reversibility of observed toxic effects. The toxicity assessment of the vaccine formulation can be done either in a) dedicated-stand alone toxicity studies or b) combination safety/activity studies with toxicity endpoints incorporated into the design of the study. This should also include an assessment of local tolerance.

4.1.2 Animal species, sex, age, size of groups

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

Where possible, the safety profile of a product should be characterized in a species sensitive to the biological effects of the vaccine. Ideally, the species should be sensitive to the pathogenic organism or toxin. The animal species used should develop an immune response to the vaccine antigen. In general, one relevant animal species is

sufficient for use in toxicity studies to support initiation of clinical trials. However, there may be situations where two or more species may be necessary to characterize the product, for example where the mechanism of protection induced by the vaccine is not well understood (for example, intranasal influenza vaccine and intranasal measles vaccine).

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

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

In general, the approximate age for rodents is six to eight weeks, and for rabbits, 3 to 4 months, at the start of the study.

4.1.3 Dose, route of administration, controls

The toxicity study should be performed with a dose maximizing exposure of the animal to the candidate vaccine and the immune response induced, such as peak antibody response. In general, dose response evaluation is not required, as part of the basic toxicity assessment and the lethal dose does not have to be determined.

However, pilot dose response studies may be conducted to determine which dose induces the highest antibody production in animal model. If feasible, the highest dose (in absolute terms) to be used in the proposed clinical trial should be evaluated in the animal model. However, sometimes the dose is limited by the total volume that can be administered in a single injection and therefore guidelines for animal welfare should be followed. In such cases, the total volume maybe administered at more than one sites using the same route of administration. Alternatively, a dose that exceeds the human dose on mg/kg bases and that induces an immune response in the animal model may be used. In such cases, the factor between human and animal dose should be justified.

The number of doses administered in the animal model should be equal to or exceed the number of doses proposed in humans. To better simulate the proposed clinical usage, vaccine doses should be given as episodic doses, rather than daily doses, the dosing interval used in the toxicity study may be reduced (e.g., 2 to 3 weeks interval) compared to the proposed clinical dosing interval. The nonclinical dosing interval may be based on the kinetics of the primary and secondary antibody response observed in the animal model. A single dose study may be performed in situations where vaccine induced antibodies are expected to neutralize a live viral vector, thus limiting the expression of the gene of interest (e.g. anti-adenovirus immune response), or when immune responses induced in animals are expected to react with species-specific proteins present in the vaccine formulation (e.g., human recombinant cytokines used as adjuvants).

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

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

4.1.4 Parameters monitored

Toxicity studies should address the potential for local inflammatory reactions, including effects on the draining lymph nodes, systemic toxicity, and effects on the immune system. A broad spectrum of information should be obtained from the toxicity studies. In-life parameters to be monitored should include daily clinical observations, weekly body weights and weekly food consumption. During the first week of administration it is recommended to do frequent measurement of body weight and food consumption, if feasible, as these are sensitive parameters indicating "illness". Interim analysis of haematology and serum chemistries should be considered within approximately 1-3 days following the first and last dose administration and at the end of the recovery period. Haematology and serum

chemistry analyses include at the minimum, an evaluation of relative and absolute differential white blood cell counts (lymphocytes, monocytes, granulocytes, abnormal cells) and albumin/globulin ratio, enzymes, electrolytes, respectively. In some cases, it may be of value to also evaluate coagulation parameters, urine samples, serum immunoglobulin classes etc. Data should be collected not only during treatment, but also following the treatment free phase (e.g., 2 weeks or more following the last dose) to determine persistence, exacerbation and/or reversibility of potential adverse effects.

At study termination, final body weights (fasted) should be obtained. Terminal blood samples should be collected and serum chemistry, hematology and immunological investigations should be done as described above, under 4.1.4 (parameters monitored). Immune response induced by the vaccine candidate should be evaluated in order to confirm the animal exposure that is also a confirmation of the choice of the relevant animal model. A complete gross necropsy and tissue collection and preservation including gross lesions and organ weights, should be conducted (annex 1, 32). Histopathological evaluations on tissues should be performed whereby special attention should be paid to the immune organs, i.e. lymph nodes (local and distant to application site), thymus, spleen, bone marrow and Peyer's patches or bronchus-associated lymphoid tissue, as well as organs that may be primarily affected due to the particular route of administration. Histopathological examinations should always include pivotal organs (brain, kidneys, liver, reproductive organs) and the site of vaccine administration. The extent of the list of tissues to be examined (i.e., from a reduced list limited to immune and pivotal organs to full list as provided in annex 1) will depend of the vaccine in question, and the knowledge and experience obtained by previous nonclinical and clinical testing of the vaccine components. For example, full

tissue examination will be required in the case of novel vaccines with no prior nonclinical and clinical experience. Therefore, the list of tissues to be tested should be defined on a case by case basis, following consultation with the relevant Regulatory Authority. Data should be reported in full as the original collection of values, and summarized.

4.1.5. Local tolerance

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

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

4.2. Additional toxicity assessments

4.2.1. Special immunologic investigations

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

or cell-mediated immune response against antigenic determinants of the host itself as a consequence of molecular mimicry (Verdier 2002; Wraith, Goldman & Lambert, 2003) or exacerbation of the disease (e.g., inactivated measles vaccine). In such cases, additional studies to investigate the mechanism of the effect observed might be necessary.

Great similarity of vaccine determinants and host molecules could cause autoimmune reactions induced by molecular mimicry (26). Therefore, any vaccine antigen which might present mimicry with a host antigen should be considered with caution, even though it is recognized that molecular mimicry does not necessarily predispose to auto-immunity.

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

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

It should be noted that observations of biological markers for autoimmune reactions are not necessarily linked to pathogenic consequences. For instance, the presence of autoimmune antibodies does not necessarily indicate the induction of autoimmune disease (36).

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

4.2.2. Developmental toxicity studies

Developmental toxicity studies are usually not necessary for vaccines indicated for immunization during childhood. However, if the target population of the vaccine includes pregnant women and women of childbearing potential, developmental toxicity studies should be considered, unless a scientific and clinically sound argument is made by the manufacturer that conducting such studies is not necessary. For a preventive vaccine, reproductive toxicity assessments are generally restricted to pre- and postnatal developmental studies, since the primary concern is any potential untoward effect on the developing embryo/foetus/newborn. The need to conduct fertility and post-weaning assessments would need to be considered on a case-by-case basis. The animal model chosen should develop an immune response to the vaccine, which is usually determined by serum antibody measurements. In addition, it is important to evaluate maternal antibody transfer by measuring vaccine induced antibody in cord or foetal blood in order to verify exposure of the embryo/foetus to maternal antibody. The route of administration should mimic the clinical route of administration. Ideally, the maximal human dose should be administered. If it is not possible to administer the full human dose, e.g., due to limitations in total volume administration or if local toxicity is observed that may result in maternal stress, a dose

that exceeds the human dose on a mg/kg bases and able to induce an immune response in the animal should be used.

To assess any potential adverse effects of the vaccine during the period of organogenesis, the pregnant animal is usually exposed to the vaccine during the period from implantation through closure of the hard palate and end of pregnancy defined as stages C, D and E in the ICH S5a document (29). Because of the relative short gestation period of most animal models used, pre-mating treatment is frequently required in order to assure maximal exposure of the embryo/foetus to the vaccine induced immune response. For a preventive vaccine, the number of doses administered depends on the response onset and duration of the response. Booster immunizations at certain times during the period of gestation may be necessary to maintain high level of antibody throughout the gestation period and to expose the developing embryo to the actual components of the vaccine formulation. Endpoints include, but are not limited to, viability, resorptions, abortions, foetal body weight, and morphology. The reader is referred to the other publications for guidance on endpoints used to evaluate potential toxic effects of the product on embryo/foetal development (29). It is also recommended that post natal follow up of pups from birth to weaning be incorporated in the study design to assess normal growth, body weight gain, nursing activity and viability. Therefore, studies should be designed to divide test groups into subgroups whereby half of the animals are subjected to C-sectioning and the other half is allowed to deliver their pups.

4.2.3. Genotoxicity and carcinogenicity studies

Genotoxicity studies are normally not needed for the final vaccine formulation. However, they may be required for particular vaccine components such as novel adjuvants and additives. If needed, the in vitro tests for the evaluation of mutations and chromosomal damage should be done prior to first human exposure. The full battery of tests for genotoxicity may be performed in parallel with clinical trials (30).

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

4.2.4. Safety pharmacology

The purpose of safety pharmacology is to investigate the effects of the candidate vaccine on vital functions. If data from nonclinical and/or human clinical studies suggest that the vaccine (e.g. one based on specific toxoids) may affect physiological functions (CNS, respiratory, cardiovascular, renal functions) other than the immune system, safety pharmacology studies should be incorporated into the toxicity assessment. Useful information on this topic can be found in the Note for Guidance on safety pharmacology studies for human pharmaceuticals (31).

4.2.6 Pharmacokinetic studies

Pharmacokinetic studies (e.g. determining serum or tissue concentrations of vaccine components) are normally not needed. The need for specific studies should be considered on a case by case basis (e.g. novel adjuvants or alternative routes of

administration) and may include local deposition studies which would assess the retention of the vaccine component at the site of injection and its further distribution (e.g. to the draining lymph nodes). Distribution studies should be considered in case of new formulations, novel adjuvants or when alternative routes of administration are intended to be used (e.g., oral or intranasal).

5. Special Considerations

5.1 Adjuvants

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

Adjuvant activity is a result of multiple factors and the immune response obtained with one particular antigen/adjuvant formulation cannot be, as a rule, extrapolated to another antigen. Individual antigens vary in their physical and biological properties and antigens may differ with regard to help from an adjuvant. Adjuvants must be chosen based on what type of immune response is desired and adjuvants must be formulated with the antigen in such a way that distribution of both is optimised to ensure presentation to the relevant lymphatic tissues. The vaccine administration route is also an important factor influencing the efficacy and safety of an adjuvant.

The effect of the adjuvant should be demonstrated in preclinical immunogenicity studies. If no toxicological data exist for a new adjuvant, toxicity studies of the adjuvant alone should first be performed. In general, assessment of new or novel adjuvants should be undertaken as required for new chemical entity (33,34,35). These data may be generated by the vaccine manufacturer or by the producer of the adjuvants. In addition to assessing the safety of the adjuvant by itself it is also important to assess whether the antigen/ adjuvant combination exerts a synergistic adverse effect in the animal model compared to the individual components (21, 22). In cases where species-specific proteins are used as novel adjuvants (e.g., cytokines), the issue of species specific response should be considered.

When evaluating the safety profile of the adjuvant/ vaccine combination, the formulation proposed to be used clinically, should be used.

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

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

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

5.2. Additives (Excipients and preservatives)

Where a new additive is to be used, for which no toxicological data exist, toxicity studies of the additive alone should first be performed and documented according to the guidelines for new chemical entities (34). The compatibility of a new additive with all vaccine antigens should be documented as well as the toxicological profile of the particular final vaccine formulation in animal models as outlined in section 4.

5.3 Vaccine formulation and delivery device

The vaccine formulation, e.g., liquid form, capsules or powder, as well as the delivery device, are factors that may have an impact on the uptake of the vaccine, its effectiveness and safety. Ideally, the delivery device and vaccine formulation tested in an animal safety study should be identical to the one intended to be used clinically. However, animal models in which clinically intended delivery devices can be used may not be available. In these instances, in order to develop an appropriate animal model, it may be necessary to conduct pilot studies to define and optimize the conditions for drug delivery in the animal model so that it can be used to assess the preclinical safety of the product.

5.4. Alternate routes of administration

When using vaccine formulations given by alternate routes of administration (e.g., intranasal, oral, intradermal, rectal and intravaginal routes), it can be assumed that their potency, relevant immunogenicity, tolerability, toxicity, and long-term safety may be different to products delivered by the parenteral route. Thus, when

different routes of administration are proposed, non-clinical safety studies may have to be conducted using vaccine formulation and/ or adjuvant alone in a suitable animal model addressing specific safety concerns associated with vaccine administration via these routes. The following will discuss issues for vaccines administered using alternate routes of administrations that may need to be considered in addition to those described elsewhere in this document.

5.4.1. Animal models

Of special consideration for vaccines administered via alternate routes should be the anatomy and physiology of the site of vaccine administration of the particular animal model chosen and its accessibility to the test article administration. For example, for intranasaly administered products, the species chosen should ideally be receptive to spray administration of the product. In general, rabbits and dogs are useful test models for use of spray devices, however their olfactory bulbs are highly protected and special techniques would be required to ensure that the test article reaches this organ. Mice and rats are useful models for intranasal administration studies, however administration of the test article is limited to droplets. Nonhuman primates may also be considered, especially if they are susceptible to infection by the micro-organism in question, after nasal administration (e.g., intranasal measles vaccine).

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

5.4.2. Dose

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

5.4.3. Endpoints

The toxicity endpoints would include those described in section 4 of this document and may include additional outcome measures that will depend on the route of administration, specific concerns associated with the particular route and target organ. For example, if concerns exist for the potential passage of vaccine components to the brain following intranasal administration, immunohistology and "in situ" methods and/or neurologic assays and examinations may need to be performed. For vaccines administered by inhalation, outcome measures may include pulmonary function tests and data on histopathology of the lungs. Considerable efforts may need to be undertaken to develop appropriate methods to address potential safety concerns associated with the use of new routes of administration.

5.4.4. Immunogenicity assessment

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

6. Specific considerations for particular types of vaccines

In addition to the testing strategies outlined in sections 3, 4 and 5, studies may be necessary to address specific safety concerns associated with particular product types using adequate in vitro and in vivo test methods. The following will discuss specific testing requirements for live attenuated and combination vaccines. For other product categories, detailed information regarding the production and control of vaccines is available in the WHO guidance documents for production and control (6), and should be consulted. For example, in recently developed guidelines for DNA (15) and synthetic peptide vaccines (17), as well as for particular vaccines such as Hib conjugated vaccine (38), the issues relevant for nonclinical testing are discussed and should be considered in the development of an appropriate design for the nonclinical study of the vaccine in question.

6.1 Live attenuated vaccines

An assessment of the degree of attenuation, and the stability of the attenuation phenotype, is an important consideration for the nonclinical testing programme of a

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

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

If the live attenuated vaccine is based on a genetically modified organism, then an environmental risk assessment may be required as part of the pre-clinical evaluation. An investigation into the possible shedding of vaccine organisms

following administration contributes to the environmental risk assessment. For all live attenuated vaccines, information on the likelihood of exchange of genetic information with non-vaccine strains may be required and suitable nonclinical tests may be designed to provide data for this purpose.

6.2. Combined vaccines

New combinations produced either by formulation or at the time of reconstitution of antigens or serotypes should be studied for appropriate immunogenicity in an animal model, if available, before initiation of human clinical trials (7,8). Combined antigens should be examined by appropriate physico chemical means to evaluate possible changes to antigen properties on combination, such as degree of adsorption to aluminium adjuvants, as well as a stability of the combination.

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

The safety of the new combination should be evaluated in an animal model on a case by case basis and especially if there is a concern that combining antigens and/or adjuvants may lead to toxicity problems (e.g., novel adjuvant).

Similar consideration for nonclinical testing will also apply to cases where a new candidate monocomponent vaccine is developed from an already licensed combined vaccine (e.g., monovalent OPV vs. trivalent OPV).

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