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Nonclinical safety assessment of vaccines: up to date applications

Aşıların klinik dışı güvenlik değerlendirmesi: güncel uygulamalar

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ABSTRACT

Vaccines have a great impact on global health. These pharmaceutical products are prophylactic agents administered to healthy individuals, involving infants and children. Therefore, it is important to demonstrate the safety of them with nonclinical studies before the start of clinical trials. Nonclinical assessment includes product characterization, both in vitro and in vivo testing of vaccines, adjuvanted vaccines or vaccine adjuvants. In vivo safety studies include pharmacology studies, pharmacokinetic studies, general toxicity studies, developmental and reproductive toxicity, genotoxicity and carcinogenicity studies, and immunogenicity assessment. These tests should be conducted in compliance with GLPs. Nonclinical studies are conducted to determine safety and appropriate dose to induce an immune response in animal models. A benefit-to-risk profile is considered for each vaccine because of many factors that affect nonclinical and clinical toxicities. Herewith, the non-clinical safety evaluation of vaccines, including toxicity testing, has been focused. Nonclinical testing requirements are an essential tool to determination of the safety and efficacy of vaccines.

Keywords: Nonclinical safety assessment, in vitro studies, in vivo studies, toxicity.

ÖZ

Aşıların küresel sağlık üzerinde büyük etkisi vardır. Bu farmasötik ürünler, bebekleri ve çocukları da kapsayan sağlıklı bireylere uygulanan profilaktik ajanlardır. Bu nedenle klinik araştırmalara başlanmadan önce bunların güvenliğinin klinik öncesi çalışmalarla ortaya konması önemlidir. Klinik dışı değerlendirme, aşıların, adjuvanlanmış aşıların veya aşı adjuvanlarının hem in vitro hem de in vivo testlerini içeren ürün karakterizasyonunu içerir. İn vivo güvenlik çalışmaları farmakoloji çalışmalarını, farmakokinetik çalışmaları, genel toksisite çalışmalarını, gelişimsel ve üreme toksisitesini, genotoksisite ve karsinojenisite çalışmalarını ve immünojenisite değerlendirmesini kapsar. Bu testler İLU'ya uygun olarak yapılmalıdır. Hayvan modellerinde immün tepkiyi tetiklemek için güvenliği ve uygun dozu belirlemek amacıyla klinik dışı çalışmalar yürütülmektedir. Klinik dışı ve klinik toksisiteleri etkileyen birçok faktör nedeniyle her aşı için bir fayda-risk profili dikkate alınır. Bu derlemede aşıların toksisite testleri de dahil olmak üzere klinik dışı güvenlik değerlendirmesine odaklanılmıştır. Klinik dışı test gereklilikleri, aşıların güvenliğinin ve etkinliğinin belirlenmesinde önemli bir araçtır.

Anahtar Sözcükler: *Klinik dışı güvenlik değerlendirmesi, in vitro çalışmalar, in vivo çalışmalar, toksisite.*

INTRODUCTION

Vaccination of healthy people against childhood or infectious diseases from the first year of their lives is a very important issue for public health. Over the years, many diseases are largely controlled by effective vaccination programs. For example, while the number of paralytic cases of polio around the world before vaccine was over 350,000 per year, the disease was eliminated with vaccination in the 1960s and 70s.

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According to 2016 data, the disease was still prevalent in 125 countries in the world and the annual number of cases with paralysis was reduced by more than 99% with 42 cases (1). Nowadays, over two billion people suffer from infectious diseases prevented by vaccinations. It is a large fact that prophylactic vaccines prevent disabilities and diseases on public for years. In addition to this, new generation therapeutic vaccines are recently used for noninfectious and chronic diseases such as cancer. However, the concerns about potential risks of overall vaccines often prevent the perception of their benefits (2). Therefore, it is very important to demonstrate the safety as well as the efficacy of vaccines. From this point of view, a process was started to identify a nonclinical assessment approach in vaccine development in the 1990s (3).

Vaccines are special pharmaceutical products that may include inactivated bacteria or virus (inactivated whole-cell), inactivated toxoid, or live-attenuated vaccine strains. Live- attenuated vaccines with long-term immune response are among the most effective vaccines against human infectious disease according to European Medicines Agency (EMEA). Live recombinant vectored vaccines are produced using bacteria or viruses and live recombinant vectors express heterologous antigens by the antigen-encoding genes (4,5). Attenuation and recombination events in live- attenuated or recombinant vaccine strains may carry risks related to the reversion of vaccine strains to virulence (6). Therefore, the attenuation mechanisms of vaccine strains should be well defined. New generation vaccines produced by recombinant DNA technology have provided better protection than some conventional vaccines and they are safer. Among these vaccines, subunit vaccines consist of purified, recombinant or engineered proteins, or peptides (7). Polysaccharide and conjugated vaccines can also be considered in this group. These vaccines differ from inactivated vaccines contain only the antigenic parts of the pathogen and they are safer than the live-attenuated vaccines. Also, nucleic acid vaccines and therapeutic vaccines currently used for immune response (8,9). In DNA vaccines, genetically engineered DNA (DNA plasmid having antigens) is used to stimulate both humoral and cellular immunity (10).

Vaccines also contain other components such as adjuvants, stabilizers, preservatives, and trace substances produced during the manufacturing process alongside highly purified antigens (11). Adjuvants are pharmacological or immunological agents included in vaccine formulations to enhance the immunogenicity of vaccine antigens. Although not all vaccines need adjuvants, many vaccines -especially live-attenuated vaccines- often include adjuvants/adjuvant systems. These components consist of heterogeneous materials such as salts (e.g., aluminum), oil emulsions (e.g., squalene), lipid A derived from lipopolysaccharide (LPS), saponin-based mixtures and oligonucleotides (9, 12) and they are not considered active ingredients (11). Adjuvants used in vaccines must be determined in keeping with the type of immune response and should be used in accordance with pharmacopoeia to avoid toxicity. The effects of adjuvants should be revealed in nonclinical immunogenicity studies (13-15). The safety of vaccine adjuvant is evaluated according to the specific vaccine in which it is used (13). Therefore, each vaccine should be evaluated individually and the safety assessment of them should be thorough and continuous.

During the production and as end-product, vaccines are tested in a number of nonclinical and clinical evaluation studies (16). Nonclinical assessments are considered as the initial step of a vaccine guiding from the laboratory tests to the clinical assessment (17, 18). For the nonclinical assessments of vaccines, several guidelines have been produced since 1997 by the major regulatory and public health agencies such as World Health Organization (WHO), the European Medicines Agency (EMEA), the International Conference on Harmonization (ICH), the USA Food and Drug Administration-Center for Biologics Evaluation and Research (FDA-CBER), and other regulatory agencies. These guidelines are shown in Table-1. In all guidelines, the general principles of nonclinical evaluations of vaccines and the regulatory authorities' expectations for new vaccines are discussed. These guidelines have a similar scope, and their nonclinical programs are with significant alignment across agencies (19). According to WHO Guideline on nonclinical evaluation of vaccines (2005), nonclinical evaluations of vaccines contain "all in vivo and in vitro testing performed before and during clinical development of vaccines" (15). The definition of preclinical evaluation in this guideline is described as "all in vivo and in vitro testing carried out prior to the first testing of vaccines in humans". When both definitions are considered, it is understood that nonclinical evaluation includes preclinical studies as well as nonclinical tests performed during the clinical trial phase.

Table-1. Guidelines for the nonclinical assessment of vaccines for human use (Modified from Sun et al., 2012 (50)

Vaccine type	Guidelines
All vaccines	EMEA, 1997. Note for guidance on Preclinical pharmacological and toxicological testing of vaccines, EMA/CPMP/SWP/465/95.
	WHO Guidelines on nonclinical evaluation of vaccines, 2005 (WHO Technical Report Series No 927, Annex 1). WHO/BS/03.1969.
	FDA-CBER, 2006. Guidance for Industry: Consideration for developmental toxicity studies for preventive and therapeutic vaccines for infectious disease indications.
DNA and vector-based vaccines	EMEA, 2001. Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal products, CPMP/BWP/3088/99
	EMEA, 2008a. Guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products.
	EMEA, 2010. Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines, EMA/CHMP/VWP/141697/2009
	WHO Guidelines for assuring the quality and nonclinical safety evaluation of DNA vaccines, 2007 (WHO Technical Report Series, No 941)
	FDA-CBER, 2007. Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indication.
Recombinant vaccines	ICH Harmonized Tripartite Guideline, ICH S6 (R1), 1997: Preclinical safety evaluation of biotechnology-derived pharmaceuticals (Addendum 12 June 2011)
Viral vaccines	EMEA, 2002. Note for Guidance on the development of vaccinia virus-based vaccines against smallpox.
	EMEA, 2007. Guideline on influenza vaccines prepared from viruses with the potential to cause a pandemic and intended for use outside of the core dossier context.
	EMEA, 2008b. Guideline on dossier structure and content for pandemic influenza vaccine marketing authorization application.
	FDA-CBER, 2010. Guidance for Industry: Characterization and qualification of cell substrates and other biological materials used in the production of viral vaccines for infectious disease indications.
Adjuvants in vaccines	EMEA, 2005. Guideline on adjuvants in vaccines for human use, CHMP/VEG/134716/2004.
	WHO Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines, 2013a (WHO Technical Report Series, TRS 987, Annex 2, 2014)

EMEA: European Medicines Agency; WHO: World Health Organization; ICH: International Conference on Harmonization; FDA-CBER: United States Food and Drug Administration

A clearly defined vaccine-specific developmental strategy is crucial to ensure the efficient and successful development before initiation of nonclinical and clinical evaluations (18-20). Similar with chemical drugs, vaccine development process typically comprises many phases. These phases are shown in Figure-1.

The nonclinical assessment of a vaccine development process is carried out in multiple stages and is a complex multidisciplinary activity (21). The vaccine components and the final vaccine product are tested for purity, sterility, potency, consistency, activity, and stability. Also, vaccines are assessed for efficacy, toxicity, immunogenicity and safety. These tests are conducted both in vitro (in the laboratory) and in vivo (in animal models), and both studies contribute to vaccine characterization and safety evaluation (12). Nonclinical assessment studies in relevant animal models are more valuable for identifying potential risks of the vaccines.

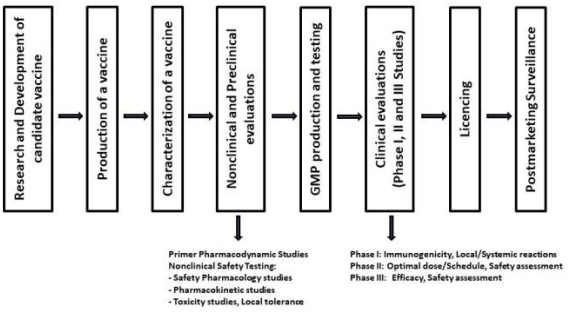

Figure-1. A vaccine development, production, evaluation and marketing process. This process carries out depending on Good Manufacturing Practices (GMP), Good Laboratory Practices (GLP), Good Clinical Practices (GCP), Guidelines of Major Regulatory Agencies and National Regulatory Authorities.

However, animal tests should be performed according to the national and international animal welfare acts, appropriate biosafety necessities and compliance with Good Laboratory Practice (GLP) (OECD Principles on GLP, 1998 (22); WHO Manual of Laboratory Methods, 1997 (23); WHO Laboratory Biosafety Manual, 2020 (24); Code of Federal Regulations 21 CFR 58, 2024 (25)). Nevertheless, there are some limitations in animal testing. The immune responses in animal models may not project human studies due to species specific susceptibility to infection by viruses, bacteria, and other microorganisms. Despite this, animal models in toxicology and pathophysiology can be used to predict human outcomes (26). As a result, there has been an increased focus on nonclinical evaluation of vaccines in recent years (2). The candidate vaccine must be tested in comprehensive nonclinical studies and appropriately designed clinical trials (21). Nonclinical assessment requirements of a candidate vaccine include:

1. Characterization of candidate vaccine (quality control testing program)
2. Pharmacodynamics studies (Primary pharmacodynamics studies: proof of concept testing and protective efficacy studies in animal models, secondary pharmacodynamics and safety pharmacology studies)
3. Pharmacokinetic studies
4. Preclinical safety testing (toxicity studies in animal models)

All these assessments play a crucial role in providing safety of vaccines and, they eliminate candidate vaccines that have inadmissible risks for clinical assessment testing on human (5).

NONCLINICAL ASSESSMENT

According to WHO, the potential toxicity of a vaccine should be assessed not only prior to initiation of human trials, but also throughout preclinical studies. Preclinical assessment is essential in moving a vaccine from the laboratory to the clinical studies and this assessment includes all test procedures such as characterization of vaccines, primary pharmacodynamics and safety testing on animals carried out prior to human clinical trials (18, 19). However, nonclinical assessment may only be needed when changes in the manufacturing process or product formulations are made or to further study potential safety concerns that may have arisen from phase I and II clinical studies or that have been reported in the literature for similar products (15).

Characterization of Candidate Vaccine

Vaccines are a unique category of pharmaceuticals, and they have to be both effective and extremely safe. The biological nature and the manufacturing process of the candidate vaccine are important factors to be considered in the plan of nonclinical assessment of vaccines. The quality, potency and safety of vaccines may vary depending on the manufacturing conditions. In case of any modification in the manufacturing process of a vaccine, the quality, efficacy and safety should be re-evaluated (15). Therefore, the manufacturing process of vaccines must be carried out in accordance with Good Manufacturing Practices (GMP).

Vaccines are derived from well-characterized materials and include disease-specific antigens such as live/attenuated viruses or bacteria, viral vector-based products, virus-like particles, virosomes, purified protein antigens (natural or produced by recombinant DNA technology), peptides, glycoproteins, protein conjugates, novel nucleic acid systems, polysaccharides etc. All these elements pose challenges for characterization (27). Occasionally, purified antigens can produce weak immune responses, so it is very important to choose an appropriate vaccine delivery system that enhances and encourages a protective immune response (21).

Some of vaccines are produced using prokaryotic or eukaryotic microorganisms (15). These organisms can be highly immunogenic and stimulate an immune response like a natural infection (21). To identify antigens against

infectious disease, information about structure of pathogen, route of entry into the body; interaction with host cells and cellular receptors, and mechanisms of pathogenicity should be identified. Any possible alteration in these organisms may affect the vaccine product and for this reason the establishment of main seed strains and seed-stocks is required for vaccine production. Using appropriate characterization methods for candidate vaccine depending on its component is very important for their clinical use. Therefore, effective physico-chemical and biological characterization methods are needed for the vaccine candidate. Also, vaccines should be tested for content uniformity (28).

For vaccine safety, it is crucial to characterize the physicochemical and functional properties of vaccine antigens and vaccine adjuvants as well as formulation and antigen–adjuvant interactions in the final vaccine formulation (27). The quality and stability of the antigen, adjuvant or adjuvanted vaccine formulation must be comprehensively evaluated before their use in a nonclinical toxicity study (29). The characterization of antigens and adjuvants used in the primary pharmacology, nonclinical safety pharmacology, nonclinical toxicology and human clinical studies should be consistent and well-documented. It is recommended that the same lots of antigen and adjuvant used in the final formulation for clinical trials should also be used in non-clinical toxicology studies (28).

Characterization of a Vaccine Antigen

It is important to monitoring specific parameters with in-process control during the process and to quantify the characteristics of the final vaccine antigen once all process stages are completed (27). Although the systems and processes used for production of vaccine antigens may vary, a number of physicochemical parameters such as size, homogeneity, purity, quantity, identity and stability should be measured for vaccine antigens. The vaccine characterization methods are based on the study of physical-chemical properties using analytical methods. For antigen characterization, physico-chemical and immunochemical techniques are used (27).

For production of protein/glycoprotein-based vaccine antigens, different expression systems are used. Multi-step purification process is required for this step. Protein analysis and characterization process for these vaccine antigens include protein structure analysis, activity, physico-chemical and immunochemical properties, protein quantity, potency and biological activity, purity/impurities and contaminants determination (30). Purity which is one of the main physicochemical parameters is used to determine the percentage of active vaccine antigens in the final bulk (27). Electrophoretic and chromatographic methods (for peptide length, isoelectric pH, size, charge, polarity etc. determination), sedimentation and light scattering analyses (for mass/size, mass/charge measurement) are used to assess the purity of recombinant-protein/glycoprotein-based vaccine antigens (27, 31).

Viral material has the propensity for particle formation and aggregation. For the investigation of these circumstances during manufacture and especially storage, analytical techniques comprising chromatographic methods such as liquid chromatography (LC), liquid chromatography - mass spectrometry (LC-MS) are used. (32). These techniques allow the studies of the entire virus or profile of the viral proteome (32). Viral proteome fingerprinting can be done by chromatography (such as HPLC), Matrix-Assisted Laser Desorption/Ionization (MALDI) mass spectrometry and gel electrophoresis (such as SDS-PAGE) techniques. For vaccines containing oligonucleotide, accurate revealing of physicochemical characteristics such as identity, purity, quality, strength, structure characterization etc. are required (32). Molecular weight and molecular sequencing are used for assurance of the identity of an oligonucleotide (33). The purity and impurities analysis of the oligonucleotide are performed with chromatographic methods (34).

Throughout the entire vaccine development process -from initial characterization to final manufacturing and testing- these technologies are invaluable. The methods used in the characterization and control of currently licensed conventionally produced vaccines are probably not applicable to new vaccine products developed using advanced technology to protect against the same infection (15). Also, specific guidelines have been improved for the production, characterization and quality control and evaluation of vaccines. These guidelines and standards are described for each vaccine by “The Expert Committee on Biological Standardization” in the WHO.

Stability Tests of a Vaccine

The stability of a vaccine refers to its ability to maintain its physico-chemical and biological

properties within defined limits throughout its shelf life. Vaccines are complex mixture and unique. Therefore, stability of each vaccine should be evaluated specifically. The stability of a vaccine has a great impact on immunization. For this reason, the potency of a vaccine is evaluated during stability studies. Also, the use of physico-chemical characteristics of a vaccine in stability evaluation allows monitoring of any changes in vaccine antigen over time (35,36).

Stability evaluation of a vaccine is a continuous process at all stages from the development of the vaccine to post-license monitoring (See Fig 1) (37,38). In the past, stability tests had been focused on efficacy of vaccines at different temperatures. This is because vaccines are very sensitive to inactivation by environmental factors such as temperature, time, handling and storage conditions (15,39). As some vaccines are oversensitive to light factors such as light also should be considered in the development of new vaccines (35). Stress testing studies that are not regularly performed as part of a stability evaluation, are used to detect the intrinsic stability of a vaccine (35). Stress testing is performed under extreme conditions such as extreme temperatures or light.

Sufficient data to elicit the stability of a vaccine entering human clinical studies should be collected during nonclinical assessment. Vaccine stability data are usually collected in two stages: Real storage condition studies in suggested storage temperature and accelerated stability studies in higher temperatures (35). In these tests, vaccine characteristics including biological activity especially potency, are determined. For licensing purposes, long-term stability data should be obtained under real storage conditions and these results should be supported by accelerated stability studies (35,38).

Potency Tests of a Vaccine

Potency of a vaccine is defined as the measure of specific ability or biological activity using a proper quantitative biological test such as laboratory tests or experimental animals (15). The immunogenicity of a vaccine is determined by potency and immunogenicity (primary pharmacodynamics) tests (See Section 2.2.1). Potency tests are based on the measure of the biological activity to demonstrate the protective immunity of a vaccine however do not guarantee that the vaccine will provide a protection in all cases. Even the well characterized, highly purified or synthetic antigens may lack the ability to activate the innate immune system. Due to the complex structure and immune response of the pathogen, the efficacy of the vaccine in potency tests may not always accurately predict vaccine efficacy. In some cases, vaccines that have passed control potency tests may not always provide sufficient efficacy (40). Therefore, potency evaluation is used to confirm the consistency of the manufacturing process, and this action is performed on vaccine lots (15). Potency tests of a vaccine is the measurement of the biological activity of the vaccine according to the well-defined reference materials with known bioactivity.

In routine potency evaluation, classical challenge tests are conducted on animals. The animals are first immunized with the candidate vaccine and then infected with the pathogen organism. The control group is only exposed to the pathogen. As a result of the infection, the percentage of animals that show specific symptoms or die in the test groups is recorded. This method has been shown to be very effective in demonstrating the potency of the vaccine. However, it needs to find alternatives to the use of laboratory animals. In addition to this, where no proper animal model exists for challenge tests, potency is based on measurement of immunogenicity with generally serological tests (15, 41). Potency tests for live attenuated vaccines generally differs from the others. In the measure of potency for live attenuated viral vaccines, the infectious titer in cell culture or chicken embryos is considered. In live attenuated bacterial vaccines, the number of colony forming unit (CFU) is measured for potency. These methods may not be adequate for vectored vaccines that express heterologous vaccine antigens and, in this case, other methods such as the quantitation of the expression of the insert should be used (15).

Standard and reference materials should be used in all processes (immunogenicity, potency etc.) within the scope of quality control test program of vaccines. Numerous guidelines and recommendations that outline the fundamental principles for the formulation and production of vaccines, characterizations of vaccine antigens and adjuvants, quality control of vaccine formulations, and antigen–adjuvant interactions are available (27). The European Pharmacopoeia for pharmacopeial requirements of vaccines is also established (15).

Pharmacodynamics Studies

Pharmacology studies as part of the nonclinical assessment of vaccine have been conducted for

many years (42). In the development of pharmaceutical products, pharmacodynamics tests are performed to detect pharmacological responses. Pharmacodynamics studies are carried out in three main categories: Primary pharmacodynamics, secondary pharmacodynamics, and safety pharmacology studies (42,43). Primary pharmacodynamic studies are generally carried out during the discovery stage of a pharmaceutical product development and not generally carried out in accordance with GLP requirements, while the other pharmacology studies are expected to be conducted to GLP standards, when their results are used for human safety testing (44). Data of the primary and secondary pharmacodynamics studies of the vaccine also contributes to the safety evaluation of the vaccine. In these studies, vaccine immunogenicity (protective efficacy) for the desirable immune response and vaccine immunotoxicity for the undesired/unexpected immune response are evaluated (45).

Primary Pharmacodynamics Studies

During a vaccine development, vaccine immunogenicity should be evaluated by primary pharmacodynamics studies (3). In vitro/in vitro primary pharmacodynamics studies are proof-of-concept testing in animals and are performed to investigate the mode of action and primary action in target system of the vaccine, while secondary pharmacodynamics studies are performed to reveal the resultant action in these systems (29, 44).

Immunogenicity data obtained from small animal species (e.g. mice, rat and ferret) are expected before clinical studies, because these studies are crucial because the ability of the vaccine to elicit an immune response cannot be fully assessed in humans without initial animal testing. Therefore, to provide evidence regarding the potential protective efficacy of a vaccine, challenge (or protection) studies with the infectious agent should be carried out in a proper animal model (46). These studies should be conducted using the strain intended for the candidate vaccine and should be involved an assessment of immune responses according to dose and dosing interval of vaccine. Immunization studies for protective effect of a vaccine conducted in animal models should be planned to evaluate related immune responses (antibody production level, class and subclass of antibody produced, duration of immune response and cell-mediated immunity) (15,47). Functional immunogenicity leading to protection such as the formation of neutralizing antibodies, immune complex formation, and interactive relation with immune cells should also be investigated in vaccinated animals (47). In determining the immunological characteristics of the vaccine, immunogenicity data generated from the animal models are useful. This data help about the dose selection, dosing (vaccination) schedules and administration routes of the vaccine to support for both nonclinical and clinical study plan (15,44). Determining the dosing schedules for vaccines, in vaccinated animals, seroconversion rate, seroprotectivity, mean antibody titers or cell-mediated immunity of the biologically active component in the vaccine are assessed apart from the primary pharmacodynamics studies (48). Immune response studies in animal models are also beneficial to document consistency of production, especially during the verification stage of a vaccine manufacturing process.

To confirm whether the animal model is suitable for immunogenicity studies, challenge studies could be used (15). It should be taken in consideration that some animal models often fail to foresee immune response and efficacy in humans, because humans and animals have different immune systems, their mechanisms of antibody induction vary depending on the origin and the immunological characteristics of the vaccine. For this reason, appropriate reference materials should be used in all processes for comparative immunogenicity assessment (49). Pharmacodynamics studies may also be planned to determine interference between vaccine antigens and live organisms (15,45,50). When the candidate vaccine consists of many defined antigen, the response to each antigen should be assessed separately (15,51). If a vaccine interacts with other vaccines, reciprocal antagonism may occur, so co-administration of two or more vaccines should also be assessed (47).

The pharmacology of an adjuvant; if used; should be evaluated by pharmacodynamics studies according to the “Guideline on adjuvants in vaccines for human use” (14) or “Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines” (29). Proof-of-concept studies are also recommended to support the use of an adjuvant in vaccine formulations. Vaccine adjuvants can induce or modify an immune response and the immunogenicity to the antigen

could be enhanced by the adjuvant (45). Therefore, relevant animal models should demonstrate the increased immune response to the adjuvant/antigen combination and ensure protection against a challenge of infectious agent (14, 52). Besides, mechanism of action of the adjuvant should also be assessed in the absence of the vaccine antigen (29). In vitro assays may provide valuable insights in understanding the mechanism of action of a particular adjuvant and may also provide precious supplementary data to animal studies. These assays are important especially when there are limitations such as species-specific differences in animal models (29). For this purpose, antigen-expressing cells, other immune system cells or complex tissue culture systems mimicking lymphoid tissue are used to evaluate the effects of adjuvants by quantifying activation parameters (45, 53).

Safety Pharmacology Studies

Safety pharmacology studies are carried out to investigate the secondary pharmacological effects, potential undesirable (adverse) pharmacodynamic and pathophysiological effects and to show any functional effects of vaccines on the major physiological systems (9,54). This assessment is conducted on a case-by-case basis (9). The mechanisms of the adverse pharmacodynamics effects are also investigated in these studies.

According to ICH Guideline S7A, three types of safety pharmacology studies are described (42).

1. Standard battery of tests: these involve the assessment of effects on especially central nervous (alteration in body temperature, motor activity, behavioral alteration, coordination, and sensory/motor reflexes), the respiratory system (changes in respiratory function) and circulatory systems. These should generally be completed before clinical trials (46,54-57).
2. Supplemental studies: These focus on more complicated systems such as gastrointestinal, renal and immune systems (55). Especially, vaccine adjuvants or adjuvanted vaccines have the potential to influence physiological functions beyond the immune system (29).
3. following up studies of standard tests (42): These studies are carried out for the characterization of adverse effects observed in previous studies, because these adverse effects on organ function are not readily determined by standard toxicological testing (55).

Research the any effects of the vaccine formulation on vital functions are not generally essential unless suggested by the authorities (57). There is a discrepancy between guidance documents about safety pharmacology studies. These studies are routinely performed according to the European guidance (47), while they may not be required according to the WHO guidance (15).

Safety pharmacology studies are conducted on intact animals, isolated organs or other test systems with relatively low costs. The implementation of in vitro, ex vivo, and in vivo preliminary tests within the scope of these studies helps with the decision on whether continuing vaccine development phase or not (55). In these studies, GLP compliance is recommended but not strictly necessary. In vivo studies should be carried out in the same animal species used for primary pharmacodynamics or other nonclinical pharmacology studies. The reasons for the selection of animal species used in pharmacology and safety assessment studies should be explained. To reduce animal use, conception should be given to inclusion of any in vivo evaluations as additions to general toxicity studies (29, 57, 58). Due to ethical reasons, 3R rules should be applied for the use of animals and further in vitro techniques should be developed (28, 59). Generally, there is a tendency to combine safety pharmacology studies with toxicology assessment (47). This incorporation provides advantages such as increasing sensitivity with the large number of animals used in toxicological studies, reducing the number of animals needed for safety evaluation and cost reduction (42,55).

Pharmacokinetic Studies

Pharmacokinetic studies that are performed during the nonclinical stage support the pharmacology studies are an integral part of pharmaceutical product development process (60, 61). While pharmacodynamic studies are conducted to determine the immune response of the organism to a vaccine or vaccine antigen, pharmacokinetics assays involve the quantitative evaluation of the time course of absorption, distribution, metabolism, and excretion of the vaccine (55, 62). These studies also play a critical role in explanation of efficacy and toxicology of vaccines as well as determining optimal dosage and formulation (63).

Pharmacokinetic studies ensure a mathematical basis to assess the time course of pharmaceutical products and their effects in the body. It is supported to perform pharmacokinetic studies on vaccines for improve their development and reduce the chances of negative health effects resulting from vaccination (50). However, vaccines do not establish a pharmacodynamics and pharmacokinetic profile except for non-antigen components of vaccines such as excipients (55,62). Since kinetic properties of antigens do not provide beneficial data for determining of the vaccine dose, pharmacokinetic studies (determining serum concentration of antigens) for vaccines are generally not required (16). However, these studies might be applicable if the vaccine contains adjuvants or excipients, because adjuvants might be distributed over the body. Pharmacokinetic studies for alone adjuvant and an adjuvant/antigen combination should be taken in consideration (14).

Seroconversion is the production of detectable specific antibodies in blood serum against the infectious agent (62). The presence of an antibody response after administration of vaccine to the organism demonstrates that an immune response has been initiated and a specific antibody becomes dominant in the serum (21). The original antigen that caused the seroconversion is no longer detectable in the blood, but the antigen-antibody immune complex is detectable. Seroprotectivity refers to the protective effect gained after immunization or after infection, measured as the percentage of vaccinated subjects who achieve seroconversion (62). After vaccination (or infection) there is no direct correlation between the magnitude of the antibody response and the rate of protection (21). Absence of the antibodies after vaccination does not mean zero protection effect. In vaccines, this effect may be mediated by cellular immunity. When determining the vaccination schedule for vaccines, seroconversion and seroprotectivity of the vaccine need to be investigated (62). Antibody production in response to vaccination is an indicator of immunogenicity, not efficacy. Nonetheless, experience on vaccines has proved that the linkage between immune response and vaccine effect is so robust even though the antibody is only part of the protective immune response. Mature antibody response is accepted for licensure (21).

Preclinical Safety Testing

Vaccines are applied to healthy people for prophylactic purposes; therefore, it is important to demonstrate the safety of them. Vaccine safety is subsequently monitored and evaluated by multiple aspects and at many levels during vaccine development process (12). For safe use of a vaccine on human, they are assessed with a number of nonclinical safety assessment studies. Safety pharmacology and toxicity assessment in vitro or in relevant animal species are required before the human clinical trials with a candidate vaccine (11). The aim of these studies is to identify the potential toxic effects (16,64). These assessments play significant roles in providing vaccine safety. The nonclinical safety studies allow the identification of potential toxicities expected in humans and eliminate vaccine candidates that have intolerable risks for human clinical trials (5, 16, 64). These evaluations include safety pharmacology studies, nonclinical pharmacokinetic studies, and general and/or special toxicity studies in animal models (44). In addition to this, in vitro and in vivo assays involve the identity, purity, and potency of the vaccine play an important role in assessing of vaccine safety (65). Seroconversion, seroprotectivity and efficacy of the active component of the vaccine are the basis for determination of the schedule for vaccination (48) (See Section 2.3.).

Toxicity Assessment

There are different nonclinical toxicology studies for the evaluation of new vaccine safety. These are basic toxicity assessments (single and repeat dose toxicity studies, local tolerance assessment) and additional toxicity assessments (reproductive and developmental toxicity study, mutagenicity/genotoxicity and carcinogenicity studies) (3). Before the human clinical studies, basic toxicity studies are considered as the minimum requirement for safety evaluations (15). These studies can ensure information to support the conclusion that it is rationally safe to continue clinical studies (52). The nonclinical toxicology studies should also allow for evaluation of local tolerance (15, 45). According to WHO Guideline, the toxicity evaluation studies of the vaccine formulation may be combined with immunogenicity or safety pharmacology studies or performed as stand-alone toxicity studies (15). However, according to the EU guidance, stand-alone toxicity studies are not generally demanded, and these studies should either be

integrated into safety studies or be performed as repeat-dose toxicity studies (16). In addition to this, nonclinical toxicity assessments are not required in the vaccine combinations with known antigens (16,66). Although the content of the EMEA guideline in the scope of toxicity assessments is somewhat different from that described in the WHO guideline and the other guidelines, all guidelines suggest a case-by-case approach to nonclinical safety evaluations of vaccines (52, 66).

In nonclinical safety studies of a vaccine, it is significant to determine both immunogenic and a safe dose in animals and to define potential target organs for toxicity studies (52). The aim of the nonclinical toxicity evaluation is to characterize the potential toxic effects of a vaccine before the human clinical trials. Achievement of the nonclinical toxicology studies depends on a lot of factors such as appropriate experimental study designs, relevant animal model, and eliciting an effective immune response (65, 67). The parameters such as the right animal species and strain, dose level and immunization schedule, the route of administration, duration and frequency of treatment and assessment of endpoints (e.g. clinical blood chemistry, antibody response and necropsy evaluations) should be considered in animal toxicology studies (15).

The use of up-to-date animal models to detect rare or particular toxicities that may appear in a specific human subpopulation is limited. However, to evaluate the nonclinical safety of the vaccine, toxicology studies using these animal models play a crucial role (52). In these studies, a single animal model is satisfactory provided that it showed a proper antibody response to the vaccine antigen. The study design should include a clinical vaccine formulation group, an antigen-alone group, an adjuvant-alone group, and a negative control group (injected with saline) (3).

The antigen concentration of the vaccine in nonclinical toxicology studies is a significant factor. For this, human equivalent dose based on the projected clinical dose should be experimented to allow the generation of dose-response curves to obtain higher safety margins (3). The number of doses planned to be administered to test animals should be equal to or exceed the recommended number of doses in humans (15). The intervals between dosing depends on species and the expected immune response, such as the antibody response profile induced by the vaccine antigens (3). Dosing intervals in the toxicity studies may be shorter (15). Although the standard application is 2–3-week intervals, more studies should be made to evaluate antibody levels in progress of time to assure inclusion of the minimal interval in study plan (3). If any adverse effects are observed during these studies, this information is used to estimate an initial safe dose and dose range for the human clinical studies (3). It is recommended that the lots of antigen and adjuvant in ultimate vaccine formulation used in the human clinical studies should be same with the lots tested in non-clinical toxicology studies. According to WHO, these lots should be produced in accordance with the GMPs (68).

Administration route of the vaccine in the toxicity studies must be same route of administration with that in the clinical studies. If the vaccine will be implemented in human using a particular device such as aerosol vaccines, the same device should also be used in the animal study (15). If this is not possible, another application route may be used with proper justification (16). If any toxic findings are obtained from the safety studies using a particular administration route, to understand of toxicity spectrum of the vaccine, using a different administration route in toxicity studies may be useful (15). The common administration routes are intramuscular, subcutaneous or intradermal routes. Although vaccines can be administered to experimental animals in these routes, there are limitations to large amounts of applications to rodents (9).

The toxicokinetic research conducted for vaccine adjuvants are one of the nonclinical tests advised by the regulatory agencies (63, 69, 70). These studies are mostly typically conducted in conjunction with toxicology studies and should comply with GLP standards (56). The systemic exposure of an adjuvant is determined by the toxicokinetic assays in animals. The assays determine the relationship between the administered dose and the time course of the adjuvant. These studies also evaluate the potential of the adjuvant to accumulate in a specific organ or tissue. In the toxicokinetic studies besides blood, other biological samples should also be collected (71). Selection of the test protocol and the plan of the study should be described according to circumstances (55, 63, 69).

Single Dose (Acute Toxicity) Studies

A single-dose toxicity study is a crucial part of nonclinical study data. According to WHO Guideline (15), in cases where the vaccine-induced antibodies are expected to neutralize a live viral vector, a single-dose study should be performed. In contrast to WHO Guideline (15), the EMEA Guideline (47) makes mention of single dose toxicity studies. This guideline indicates that data from at least one animal species should be obtained, and these studies should be performed with a dose that provides an adequate safety margin relative to human dose.

In many situations, data from the single dose toxicity studies is available from the repeat-dose toxicity studies. These data are also available from animal immune response studies or safety pharmacology studies on the condition that histopathology of target organs is included (16). Therefore, generally when a repeated dose toxicity study will be available, stand-alone single dose toxicity study is not performed (9). Notwithstanding, single-dose toxicity studies are valuable in many situations. These studies can ensure safety and preliminary tolerability of the vaccine formulation and evaluate the acute effects of the vaccine (3,16). These studies may be important where antigens may have significant pharmacological effect and where the immune response induced by the first vaccination significantly changes reactions to subsequent vaccination (9).

Rodents are usually used in vaccine single dose studies (9). In these studies, the administration route and dose should reflect the clinical use. If toxicity findings are determined in these studies, the dose-response relationship should be characterized (72).

Repeated Dose (General Toxicity) Studies

The main studies supporting the safety profile of vaccines are repeated dose toxicity studies (9). The repeated dose toxicity studies are very important to assess multiple-doses vaccinations suggested for immunizations of humans (52). For vaccines that with require multiple doses application in the clinical use, a repeated dose toxicity study is generally required in one animal species. Although some vaccines are administered only single dose in clinical use, repeated dose toxicity studies are strongly recommended for these vaccines (16,73).

The design of these studies was defined in WHO Guidelines (15), and it was planned to use of the repeated dose toxicity study design for pharmaceutical products as experimental model for these products. However, vaccine specific issues such as in determination of experimental design, selection of dose levels, treatment period, pharmacodynamics, monitorization, follow-up period and a list of histopathology tissues should be considered.

Appropriate control groups should be included in this study design in order to assess the reversibility of possible adverse events and to investigate possible delayed adverse effects. In these studies, it must be taken into consideration whether the need for placebo or vehicle groups, solely adjuvant and antigen groups, etc. (9). According to the US, EU and Japan regulations, at least one additional dose, relative to the clinical trial should be added into this type of study design because the number of administrations in the toxicity study should exceed the number planned for human administration to provide the safety of the dosing schedule (52). This is called to as the (n+1) rule and this means that at least one more application is required as in the recommended clinical scheme (9, 52). The selection of animal species in these studies should be carefully evaluated on a case-by-case basis. The administration routes and doses of vaccines should reflect the clinical use. The dose administered to animals depends on the planned clinical dose and the expected immune response induced by the vaccine. Vaccines should be administered as 2–3 weeks' interval, rather than daily, doses according to WHO guideline (15). The EMEA Guideline also accepts the proposed episodic dosing using 2–3-week intervals (47). Thus, a clinical immunization schedule is simulated in animals with 4 administrations at intervals of 3 weeks. Repeated vaccination protocol may result in an increasingly immune response.

If applicable, single human dose (mL or mg/bw) should be administered to animals. When it is not possible, the maximum applicable dose that exceeds the human dose that induces immunogenicity in the animal should be administered. Instead of this, it may be feasible to apply the total volume to multiple sites using the same administration route. However, in certain situations that are poorly known of antibody levels or other intended immune responses, to justify the minimal interval in study design, the primary and secondary immune responses may

be evaluated over an extended period with further studies (52).

According to WHO Guideline, a wide range of information such as systemic and toxic effects on the immune system and local inflammatory reactions (See Section 2.4.2.), may be obtained from the repeated dose toxicity studies. Clinical monitorization should include general health, weekly body weights, weekly feed consumption and body temperature (52). After the dose administration, interim data analysis of serum biochemistry and hematology parameters should be carried out. Local toxicity should be evaluated prior to the vaccination and routinely day-to-day following the vaccination until the local reaction is resolved (74). At the end of the study, a gross necropsy and complete tissue histopathology are recommended. Histopathological assessment should be done on especially immune system organs and target organs. Also, other organs that may be affected due to the administration route and organs on the site of vaccine administration should be assessed histopathological. In case of the new vaccine products, whole tissue examination is required (15, 52).

Local Tolerance Assessment

Local tolerance assessment could be carried out either as a part of the repeated dose toxicity study or as a stand-alone study according to WHO Guideline (15). The aim of the local tolerance studies is to observe tissue reactions at the administration site and to evaluate with histopathology (9). Local tolerance should be evaluated at sites into contact with the vaccine antigen due to the route of administration and at sites incorrectly exposed to the vaccine (e.g. eye exposure during administration by aerosol vaccine) (15, 74).

Vaccines are commonly administered by intramuscular, subcutaneous or intradermal routes, and local reactions at injection sites are not all that infrequent in clinical use. Local toxicity should be assessed using a quantitative grading system such as Draize test as a toxicological standardization method to study irritation and toxicity of substances applied to the skin or the eye (9, 52, 75). If significant reactions are observed, follow-up studies may be conducted to examine the persistence of vaccine antigen or adjuvant at the administration site. In case of a new vaccine product, this assessment can be included in the repeat-dose toxicity studies (9). In some instances, a stand-alone study may be preferable. For example, if the repeated dose toxicity study was carried out in the mice; local tolerance assessment may be performed in rabbits as a stand-alone study.

Adjuvant frequently produce local reactions, and therefore, the adjuvant-only group should be included in the study design to assess the contribution of the adjuvant to local reactions. Symptoms and local reactions such as redness, pain, swelling, granuloma formation, abscess, necrosis and regional lymphadenopathy can be seen in local tolerance studies depending on the severity of the tissue reactions (9). The pathologist should differentiate healthy tissue responses and undesired pathological changes in the tissue as response to the injection of the vaccine. C-reactive protein (CRP) which is a sensitive marker of inflammatory changes in humans and some animal models is an acute phase response protein involved in complement activation. After immunization with vaccines that produce local reactogenicity in clinical studies, it was shown that CRP levels were raised. Therefore, it is thought to be a useful biomarker in nonclinical local tolerance studies (76).

Reproductive and Developmental Toxicity Studies

Data on reproductive function for vaccines are generally not necessary. Histopathological findings obtained from toxicity studies can provide sufficient information about the integrity of the reproductive organs (16). Besides, for prophylactic vaccines, reproductive toxicity evaluations are usually limited to pre and postnatal developmental studies to detect any potential undesirable effect on the developing embryo, fetus or newborn (15, 52). In order to verify exposure of the embryo or fetus to maternal antibody in the animal model chosen, maternal antibody transfer should be evaluated by measuring vaccine-induced antibody in cord or fetal blood. The administration route of vaccine should be same route within the clinical use and the maximal human dose should be applied to the experimental animals. If it is not possible, a dose that exceeds the human dose on mg/kg basis and is able to induce an immune response in the animal should be used (69, 77).

According to ICH S5 R2 (77), the gestating animals should be exposed to the vaccine until the end of their gestation period to assess any potential adverse effects of the vaccine during the period of organogenesis. In order to assure

maximal exposure of the embryo or fetus to the vaccine-induced immune response, due to the relatively short gestation period of most test animals used, pre-mating exposure is required. The number of doses applied depends on the time of onset and duration of the response. Booster immunizations may be essential at particular times during the gestation period to expose the developing embryo to the components of the vaccine formulation and to maintain a high level of antibody throughout the gestation period. In this study design, end-points include viability, fetal body weight and morphology, resorptions and abortions but are not limited to them. In addition to this, it is also suggested that a period of postnatal follow-up of pups from birth to the end of breastfeeding be included in the study design to evaluate especially normality of growth and viability. Therefore, these studies should be planned with experimental groups divided into appropriate subgroups (69, 77).

Mutagenicity/genotoxicity and Carcinogenicity Studies

Genotoxicity and carcinogenicity studies for the final vaccine formulation are not needed according to the EMEA Guideline (47), however these tests are required for new adjuvants and additives. If needed, prior to human clinical trials in vitro tests for identification of vaccine-induced mutations and chromosomal damage should be carried out. Whole genotoxicity tests may be performed in parallel with clinical studies (78).

CONCLUSION

One century after Spanish flu killed millions all over the globe, Covid 19 respiratory virus from the same family as some smaller epidemics from last 20 years spread quickly and caused pandemic. It is known that as of today, 17 COVID-19 vaccines have been developed and 13 marketed (79). When examined the EMA reports for market authorization of COVID-19 vaccine, it is observed that EMA assessment of the nonclinical studies consisted most frequently of comments related to study design, species selection and missing data (80). However, it appears that all steps of the vaccine development process, including their nonclinical evaluations, are also valid for these vaccines. Analysis of historical data connected to epidemics, pandemics, and vaccine development process showed three main components connected to science and society: the start of pandemic, vaccine development process including the supply process, and post pandemic challenges. Developing pandemic emergency plans against such pandemic situations in the future should be a top priority.

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