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CHAPTER

27

Preclinical Toxicology of Vaccines[1]

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OUTLINE

INTRODUCTION TO VACCINES/ ADJUVANTS FOR THE PREVENTION OF INFECTIOUS DISEASES

Vaccines are biological preparations that augment immunity to targeted diseases. These biological preparations stimulate the recipient's immune system to recognize targeted aspects of infectious organisms as foreign and generate host mechanisms to control or eliminate them. Additionally, they evoke mechanisms to form an immunological memory of the antigen(s), which provides efficacy against future infections by the same or similar organisms.

Vaccines are created from inactivated or attenuated organisms, or are derived from purified or recombinant subcomponents of these organisms. They provide antigens that may be incorporated into vaccines composed of peptides, proteins, and polysaccharides. They may also be indirectly introduced to the host immune system through recombinant DNA plasmids or chimeric virus vectors. Inactivated vaccines are killed through the use of heat or chemicals, whereas attenuated vaccines contain live, less virulent organisms. Often these vaccines are derived from live viruses that have been cultured under conditions that disable their pathogenic

[1]Disclaimer: The findings and conclusions in this chapter have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

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properties. Attenuated vaccines often produce a durable immunological response and thus are preferred for many classes of infectious agents. Subcomponents of microorganisms may also be used as antigens in vaccines. For example, toxoid vaccines are made from inactivated toxic components and offer protection from the effects of the infection. Additionally, fragments or subunits of an attenuated or inactivated microorganism can also be used as the basis of an antigenic response to a vaccine. Subcomponents may also be used for other purposes. For example, poorly immunogenic components of microorganisms can be improved by their conjugation to proteins that typically are toxins. This approach is often used in conjunction with polysaccharides, which form the outer coat of some infectious bacteria such as *Haemophilus influenzae* type B vaccine. Immunization with DNA plasmids and virus vectors involves vaccines that encode an antigen protein that are subsequently expressed within cells of the recipient following administration of the vaccine.

Monovalent vaccines are designed to provoke an immune response to a single antigen or microorganism. Multivalent or polyvalent vaccines are meant to evoke immune responses to several antigens or microorganisms; however, when various antigens are combined, both synergistic and inhibitory interactions are potential outcomes in terms of the immunological response.

The process of vaccination introduces an external substance to the host immune system, which induces or increases responses to specific antigens with sufficient vigor to provide levels of immunity to prevent the onset of disease and protect the host against the future risk of infectious disease. Responses to vaccines follow a complex and coordinated set of physiological and immune-based reactions that are tightly controlled and involve different cell types and biochemical intermediates.

Both antibody and cell-mediated responses may occur following immunization with various vaccine antigens, and are significantly influenced by the type of adjuvant used in the vaccine product. Host responses to the antigens within vaccines encompass adaptive humoral and cell-mediated immune responses and innate immune responses. Antigen-presenting cells (APCs), B cells, and T cells are initially involved. Vaccine proteins and peptides as presented by APCs interact directly with T-cell receptors that recognize the specific amino-acid sequence in association with class I or class II major histocompatibility complex (MHC) receptors, and humoral antibody production is mediated by B cells. Humoral responses include both neutralizing and nonneutralizing antibodies that involve complement-dependent and independent mechanisms and may involve T-cell dependent interactions with helper T cells and CD8+ dependent lytic and soluble-factor activities.

Vaccine-induced effectors of immunity are typically antibodies produced by B lymphocytes. Other potential effectors of immunity, such as cytotoxic CD8+ T lymphocytes, are also involved. The activities of these effectors are mediated by regulatory T-cells (Treg), which maintain immune tolerance but represent only 5–10% of the peripheral CD4 T-cell population. These cells serve to inhibit immune responses that are potentially harmful by inhibiting or increasing Th1 or Th2 activity. Treg activity is believed to play a role in controlling autoimmune diseases that could possibly arise from wayward responses to the antigen contained in vaccines through antigen-spread response. An interaction between vaccine antigens and adjuvants with Treg is likely but remains unclear and requires further research [1]. Both the generation and maintenance of B and CD8+ T cells are governed by the activity of CD4+ T helper lymphocytes, and these cells are frequently subdivided into T-helper 1 (Th1) and T-helper 2 (Th2) subtypes.

Antigens can be recognized by an antibody or T-cell receptor; however, not all antigens evoke a sufficient immune response by themselves to make them suitable vaccine components. To overcome the limitations of weak antigens, various changes are sometimes made to the vaccines. These may take the form of conjugations to the antigen itself and/or enhancement of the immune response by the inclusion of additional vaccine components such as adjuvants. For example, the ability to elicit an immune response to the antigenic components of *Streptococcus pneumoniae* in a heptavalent and triskaivalent vaccine was increased by conjugation to proteins such as diphtheria proteins. Additionally, the response to various antigens is affected by various factors, such as dose and concentration of the antigen, quantity and nature of the adjuvant, time between inoculations, and route of exposure.

Following the inoculation with a vaccine, primary and secondary immune responses occur. The schedule between injections of the vaccine can be an important determinant of the immune response, and may vary among different vaccines. After the initial primary exposure and immune response, subsequent or secondary exposures are mediated by specific populations of cells, namely short- and long-lived antibody secreting plasma cells and memory B cells.

SPECIAL TOPICS

Adjuvants

To evoke effective immune responses to a vaccine, a variety of adjuvants (chemical and biological additives) may be used [2] (Table 27.1).

TABLE 27.1 Adjuvants and Their Impact on Vaccination

Adjuvant	Effect
Alum	Denature protein
Oil (mineral)	Antigen depot formation
DEAE dextran	B-cell mitogen activation
Cholera/enterotoxin	Mucosal stimulation
Cytokines	Increase cellular immunity response
CpG	Activate CMI

Edelman [3] and Griffin [2] classified adjuvants into two groups:

1. Substances that increase the immune response to the antigen, and
2. Immunogenic proteins that modify T-cell activities.

To enhance uptake by antigen-presenting phagocytic cells, protein antigens will be denatured and precipitated by alum adjuvant [4]. When an antigen depot is created, for example by an oil-based adjuvant, slow release of the antigen occurs over a period of weeks and evokes strong immune reactions [5]. B-cell stimulation may also produce enhanced antibody responses and may be achieved by using DEAE dextran [6] or bacterial toxins [7]. Adjuvants may target innate responses that are necessary to activate specific pathways of acquired immunity [2]. Increased activity of Th1 cells, resulting in enhanced cell-mediated immunity (CMI) through selective activation of innate immunity, mediated by toll-like cell surface receptors could be caused by microbial CpG adjuvant [8].

Aluminum Adjuvants (Salts)

Aluminum-based adjuvants are well established and the most widely used, although the basis of their action remains unclear. It has been postulated that aluminum-based vaccines may function in various ways including the creation of a depot that maintains presentation of the antigen, stimulation of APCs, formation of particulate antigens from otherwise soluble antigens that increases the immunological response, and pharmacological effects mediated through the inflammasome NALP3 [9–13].

In spite of the fact that alum-based vaccines are generally well tolerated, these adjuvants may produce granulomas after subcutaneous or intradermal injections, adverse effects that are not associated with the intramuscular route of injection [14].

A number of vaccines in current use contain aluminum adjuvants [15]. Although they have less adjuvant activity than more recently developed adjuvants, their extensive human experience makes them useful and a frequent choice for vaccine candidates. The commonly used aluminum adjuvants are available in a variety of forms, such as aluminum phosphate ($AlPO_4$), aluminum hydroxide ($Al(OH)_3$), and potassium aluminum sulfate ($KAl(SO_4)_2$). The term alum specifically refers to potassium aluminum sulfate, although it may be used in a broader context to refer to other aluminum salts. The elemental aluminum content of licensed US vaccines is limited to 0.85 mg per individual dose of vaccine [15]. Aluminum salts may remain at the site of injection for long periods of time [16] and some portion of the aluminum salt is internalized by dendritic cells [17]. Nevertheless, they do undergo biodistribution and excretion over an extended period of time [18].

Despite aluminum salts having an extensive record of experience and safety, they are not ideal adjuvants. A significant problem is a potential lack of consistency in the adsorption of antigens, as different lots and brands of the same type of aluminum salt can demonstrate an inconsistent adsorptive capacity [19]. Furthermore, a potential exists for the exchange of protein antigens adsorbed to aluminum salts for interstitial proteins after injection [20–22].

The variation in adsorptive capacity and in situ interactions is likely due to the number of chemical forces binding the antigens to the aluminum adjuvants. This binding can involve a variety of factors including electrostatic bonding, hydrophobic interactions, van der Waals forces, and hydrogen bonding, and the strength of these depend on the charge on the aluminum salt and protein antigen, the physical structure of the aluminum salt, and the pH and buffer used [16,23–27].

Typically, aluminum salts induce local redness and swelling at the injection site [28], but these toxicities are readily tolerated. Local inflammation after the intramuscular injection of aluminum salts is thought to occur as the material migrates into the subcutaneous space following the needle track created on injection of the vaccine [3]. Additionally, nodules that may occur after repeated injections of these adjuvants are associated with the subcutaneous route of injection [29].

However, reports of adverse clinical findings regarding the aluminum containing adjuvant $Al(OH)_3$ producing macrophagic myofasiitis (MMF). Beginning in 1993, an increasing number of cases were reported of unusual infiltrations of skeletal-muscle connective-tissue structures by nonepithelioid histiocytic cells [30]. Patients tended to exhibit chronic myalgia in their affected limbs, and a cluster of findings presented a more coherent picture that associated MMF with aluminum salts. These cases exhibited some common characteristics:

1. The site of macrophage infiltration was focal and typically restricted to the site of injection.
2. Muscle damage was almost always absent.

3. The infiltrates of macrophages formed well-delineated sheets of histocytes.

These findings led to the conclusion that MMF is the result of long-term persistence of aluminum hydroxide at the site of injection of the vaccine [31]. The underlying causes of this human toxicity remain unclear and may be related to impaired elimination of aluminum or genetic dispositions to inflammatory disease. With respect to the latter, Authier et al. [32] examined whether differences in Th1- or Th2-biased immunity could influence the expression of MMF in a rodent animal model. These authors found that Lewis rats with a Th1-biased immune response differed in their reaction to aluminum hydroxide adjuvanted vaccine from Sprague–Dawley rats, which have a more balanced Th1/Th2 immune response. Lewis rats demonstrated significantly smaller MMF lesions than Sprague–Dawley rats. In another study, monkeys given diphtheria–tetanus vaccines containing aluminum adjuvants were found to have varying degrees of macrophage aggregation at the site of injection, although no evidence of either behavioral or muscular weakness was evident [33]. A WHO meeting on the issue of MMF highlighted the need for more research on this topic [34].

Newer, recombinant or synthetic antigens for vaccines are generally less immunogenic than older live, killed, or attenuated whole organism-based vaccines. This has resulted in the development of more powerful adjuvants to compensate for the potentially diminished immune response. Nevertheless, alum remains the major adjuvant used in vaccines used to immunize humans. Alum has the propensity to induce effective levels of antibodies mediated by Th2 responses, but has little capacity to stimulate cellular responses mediated by Th1 mechanisms. The latter is an important aspect of immunity for some newer efforts in the development of vaccines [35]. Additionally novel adjuvants address the need to develop more powerful antibody responses in human populations with insufficient responses to vaccines using alum, such as the newborn, elderly, and immunocompromised individuals. They also reduce the amount of administered antigen (antigen sparing). Although a number of recently developed adjuvants clearly demonstrate the potential for increased immunogenicity, concerns about their safety remain [36–41]. Adjuvants may be classified in various ways reflecting various properties (see Table 27.1).

TOXICITIES ASSOCIATED WITH VACCINES

Vaccines typically produce various adverse clinical effects, such as inflammation and pain at the site of injection, malaise, fatigue, and slight febrile responses. These may have their counterparts expressed in toxicity studies, such as infiltration of inflammatory cells at the site of administration, decreased food consumption, loss of body weight, or elevation in body temperature. These adverse effects reflect the activation of various components of the immune system, and will vary with the specific nature of the vaccine antigen and/or adjuvant. Similar to naturally occurring infections, the administration of a vaccine results in the activation of cells regulating immunity and the resultant inflammation is accompanied by the release of various proinflammatory cytokines and frequently evokes an acute-phase response. For example, van der Beek et al. [42] reported that after the administration of an attenuated yellow fever vaccine to healthy human subjects IL-6, C-reactive protein (CRP), and fibrinogen were found to be elevated in blood samples. Other similar studies have revealed increases in the blood levels of various cytokines and acute-phase reactants involved in immune and inflammatory responses. Reinhardt et al. [43] observed increases in β2-microglobulin after administration of a yellow fever vaccine and, additionally, Hacker et al. [44] found increases in plasma levels of tumor necrosis factor (TNF) after administration of this same vaccine.

The expression of these inflammatory cytokines and their entry into the bloodstream contributes to the expression of systemic manifestations of toxicity, like fever or malaise, which are sometimes observed after the administration of vaccines in clinical populations. In addition, other physiological effects are not well characterized and require further investigations to determine their impact on overall safety. For example, Liuba et al. [45] reported decreases in flow-mediated dilatation responses indicative of altered arterial endothelial function when measured at the brachial artery in 8 human subjects, which persisted for 2 weeks following the administration of an inactivated trivalent, split influenza vaccine. Changes in the arterial response to hyperemia were accompanied by small increases in CRP and fibrinogen levels, which were considered to be indicative of a systemic inflammatory response to the vaccine. Dilatory responses to sublingual glyceryl trinitrate and carotid intima-media thickness as measured by external ultrasound were not altered. Similarly, Hingorani et al. [46], in a small number of human subjects, found that after the administration of an attenuated capsular polysaccharide vaccine of *Salmonella typhi*, significant dysregulation of arterial endothelial function occurred in both resistance and conduit blood vessels that was accompanied by a systemic inflammatory response characterized by elevations in white blood cell count and serum levels of IL-6 and IL-1 receptor antagonist. Beyond influences on cardiovascular physiology, changes in underlying cytokine levels were reported to be factors in alterations of negative mood affect following administration of the *S. typhi*

vaccine [47,48], which may be linked to the direct influence of the inflammatory actions of vaccines on malaise, lethargy, and impaired cognitive ability sometimes observed in clinical populations.

Rarely, more serious adverse events are associated with the administration of vaccines. In many instances, it has not been possible to demonstrate a definite link between the vaccine and serious, significant toxicities.

Given the small amount of material administered in vaccines, direct local or systemic toxicity is extremely rare. More commonly, toxicities associated with vaccines arise from various factors involved in the inflammatory events that are an intrinsic part of the response to the administered antigen and/or adjuvant. Additionally, vaccines may contain excipients and preservatives, including antibiotics, that may be linked to these toxicities.[2] These additional components serve various purposes. For example, some chemicals are added during production to prevent bacterial growth or remain from the manufacturing process (extraneous proteins like egg proteins in influenza vaccines or formalin, which is found in trace amounts in several vaccine products).

Vaccines are frequently given by intramuscular injection. In addition to the toxicities caused by the vaccine components, the trauma caused by the injection introduces histological changes at the site of injection that must be considered relative to the picture of any inflammation caused by the vaccine. Thuillez et al. [49] summarized the findings of seven studies that were conducted using rats, mice, and rabbits and single or repeated injections of saline. Mice were injected in the right and left gluteus-medium muscle; rats in the left and right gluteus medium or left and right quadriceps femoris muscle, and rabbits in the dorsolumbar muscle. Mice were given 0.05 mL, while rats were given 0.2 mL and rabbits 0.5–1 mL. The authors reported that at 2 days after intramuscular injection, the lesions consisted of mainly infiltrations of inflammatory cells consisting of neutrophils or heterophils, lymphocytes and macrophages, hemorrhage, myofiber degeneration, and/or muscle necrosis. By day 10 following injection, the site contained reduced numbers of inflammatory cells along with histological evidence of healing including regeneration of myofibers and fibrosis. These findings are consistent with local, minimal trauma.

Intramuscular injections of vaccines that include alum show a similar histological picture. Verdier et al. [33] investigated the local histological effects of two aluminum-containing vaccines in monkeys after a single intramuscular injection at 3, 6, or 12 months. In these investigations, two groups of monkeys were immunized with either diphtheria–tetanus vaccine adjuvanted with aluminum hydroxide or aluminum phosphate. At 3 months, aggregations of macrophages accompanied by lymphocytic infiltrations were found at the site of injection and one monkey given aluminum hydroxide was found to have a cyst-like structure lined with macrophages and fibrocytes. Later, histological examination revealed a minimal number of lymphocytes with or without focal fibrosis in the animals given aluminum phosphate, which greatly diminished in 1 year. In monkeys given the vaccine containing aluminum hydroxide, aggregates of macrophages were evident in three of four animals and remained at 1 year.

Additionally, isolated examples of toxicities or enhanced disease in association with vaccination are known or suspected. In some aspects, these cases appear to mimic the course of increased disease severity, or adverse events due to natural infections. The most well-established examples of increased disease severity occur with respiratory syncytial, dengue, and measles virus infections. Children immunized with formalin-inactivated respiratory syncytial virus (FI)-RSV or RSV G vaccines were infected with RSV. This infection was associated with enhanced disease and pulmonary eosinophilia that was believed to be due to an exaggerated memory Th2 response [50–56]. Animal models of respiratory syncytial virus infection have suggested various mechanisms as a causal role, including sensitizing antibodies to untoward sites, unfavorable T-cell responses, or overexuberant immune responses involving cytokines or interleukins [57].

Another serious potential toxicity infrequently associated with vaccines is autoimmune disease. In this regard, three different mechanisms may be at work, namely molecular mimicry, epitope spreading, and autoimmune dysregulation. The incidence of autoimmune-induced disease is low, and in many cases cannot be reliably associated with the administration of vaccines. Although no unequivocal associations are known, various possible pathogenic mechanisms exist. Molecular mimicry is the result of an immune response to shared epitopes between antigens of the host and antigenic components of the vaccine. To assess this potential toxicity, protein sequences may be screened in computer base searches of amino-acid structures between antigenic protein components and known protein structures. Another possible mechanism for the autoimmune phenomenon is epitope spreading. Three different types of this are believed possible: shared identical amino-acid sequences between peptides and/or proteins, homologous but nonidentical amino-acid sequences, and epitopes on dissimilar chemical structures such between DNA and peptides or carbohydrates and peptides.

[2] http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/appendices/B/excipient-table-1.pdf.

http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/appendices/B/excipient-table-2.pdf.

Although the immune response to the unintended antigen may be indirect and of lower affinity or avidity, it could theoretically be of sufficient strength to provoke antibody-mediated cytotoxicity by activating complement or cell-mediated signals. Molecular mimicry of T cells differs from that mediated by antibodies. Mimicry for T cells is a type of immune degeneracy in which T cells recognize and respond to untoward antigens. T cells may exhibit epitope spreading as a response, which is not directed at the original epitope, but as recognition of epitopes in target-tissue proteins expressed in the inflammatory process caused by the vaccine. Additionally, other theoretical mechanisms exist. These include activation by superantigens of a large fraction of T-cell populations and induction of inflammatory cytokines and costimulatory molecules. However, there is currently a lack of in vivo evidence that molecular mimicry is associated with vaccines, although it remains an issue of concern.

TOXICOLOGY STUDIES FOR VACCINES (ADJUVANTS)

Types of Study and Their Endpoints

FDA regulations for preclinical toxicology studies of vaccines require the components (eg, antigens and adjuvants) to also be tested for any adverse effects. These studies should follow good laboratory practice (GLP)[3] guidelines as described in the Code of Federal Regulation (CFR) 21 [58]. In general, there are five types of toxicology study:

1. Single and/or repeat dose
2. Reproductive and developmental
3. Mutagenicity
4. Carcinogenicity
5. Safety pharmacology (normally included in the repeat-dose toxicity study if needed)

Single- and/or Repeat-Dose Toxicology Studies

Developing a new vaccine requires preclinical testing for any adverse effects (local or systemic) of the test article. Depending on the stage of vaccine development, single and/or multiple dose, dose response, and/or time response studies should be conducted.

[3]GLP system means the organizational structure, responsibilities, procedures, processes, and resources for implementing quality management in the conduct of nonclinical laboratory studies [58]. Part 58 in these regulations includes the specific GLP requirements for both in vivo and in vitro studies. Parts 11 and 809 of CFR 21 explain the GLP requirements for handling the toxicology study records and the requirements for diagnostic products for human use, respectively.

Species selection for any study should be based on the desired clinical immune response(s). For example, C57BL/6 mice are used to replicate Th1 cellular immune responses [59]. An alternative animal model is the rabbit, which is used to reproduce humoral immune responses. Other selection criteria, such as anatomical and physiological relevance to humans, may be considered. For studying intracutaneous or topical vaccines, the minipig is considered a good model [59]. The baboon was used to investigate a novel adjuvant for intranasal immunization because of its physiological and pharmacological similarities with humans [60]. For more specific investigations, such as RSV vaccine, hamsters [61] are sometimes used. Animal models for vaccine preclinical toxicology studies will be discussed in more detail later in this chapter.

Different vaccines and/or adjuvants may require different approaches for immunogenicity testing. Enhancing IgA responses might be more appropriate for mucosal vaccines development [62–64]. T-cell-mediated responses may play a key role in the vaccines' immunogenicity just as or more important than the humoral response (see "Introduction" section).

Preclinical toxicology studies should be carefully designed to include not only the relevant species, but also an appropriate number of animals (eg, 5 rabbits or 10 mice/sex/group for both main and recovery groups), route of administration of the test article (normally the same as the intended clinical route), dose level (same as the intended clinical dose), and number of doses ($N+1$, where N=number of clinical dose(s)). If the number of doses is not $N+1$, then the number employed should be justified. The number of animals in each group should be adequate to ensure reliable statistical analysis of the data can be performed, with sufficient statistical power to evaluate potential differences [65].

The study design should include all treatment groups and should include testing of the vehicle, adjuvant(s), and the antigen. Table 27.2 is an example of simple experimental design.

Test and Control Article Characterization (21 CFR Part 58.105 [58])

Identity, strength, purity, and composition should be determined for each batch of test article. Methods of synthesis, fabrication, or derivation of the test and control articles should be documented. Marketed products should be characterized by their labeling.

Stability of Test and Control Articles (21 CFR Part 58.105 [58])

Stability of the test and control articles should be determined before study initiation or concomitantly according to an approved standard operating procedure,

TABLE 27.2 Example of Experimental Design

Group Number	Identity of Group	Total Dose Volume (mL)	Dose Concentration (μg/mL)	Number of Animals/ Sex	Number of Animals Euthanized/Sex			
					Core Study Animals		Recovery Animals	
					Day	Day	Day	Day
1	Vehicle	**0.5**	Same as clinical	5 rabbits				
2	Adjuvant(s) + vehicle	**0.5**	Same as clinical	5 rabbits				
3	Antigen + vehicle	**0.5**	Same as clinical	5 rabbits				
4	Antigen + adjuvant(s) + vehicle	**0.5**	Same as clinical	5 rabbits				

which provide for periodic analysis of each batch. Stability of the test article ensures delivery of consistent concentrations of the active materials. This, in turn, ensures the consistency in the immune responses in nonclinical/clinical studies.

A preclinical study protocol should be written following the instructions in 21 CFR part 58.120 [58]. The preclinical laboratory study should be conducted in accordance with the protocol. All protocol amendments and deviations should be included in the final report. The details for reporting of nonclinical laboratory study results are included in 21 CFR part 58.185 [58]. All toxicology studies should be included in the package of the investigational new drug (IND) application.

Toxicology studies normally include the following endpoints:

Cage-side and clinical observations: Mortality, morbidity, general health, and any signs of toxicity should be monitored on a daily basis. Evaluation of skin and fur, eye and mucus membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor and behavior, should be recorded on a daily basis, or once weekly. Most of the time there are no, minimal, or mild changes in the animals' health due to vaccine treatment. Changes (if any) in animals' health due to test-article treatment could be serious and require immediate attention, or in rare cases require termination of the animal. Including recovery groups in the study will help to determine whether these changes are recoverable over time or not.

Food consumption and body weight: Changes in food consumption and body weight could be an indication of an adverse effect of the test article. Physiological events that are triggered as responses to the ingestion of food are important episodic signals [65]. Initially the brain detects, via sensory input, the amount of food ingested and its nutrient content. Specialized chemo- and mechano-receptors that monitor physiological activity are located in the gastrointestinal tract. They pass information to the brain mainly via the vagus nerve [66]. This afferent information constitutes one class of "satiety signal" and forms part of the preabsorptive control of appetite.

Appetite is controlled by chemicals released by gastric stimuli or by food processing in the gastrointestinal tract [67]. Changes in food consumption might be caused by many of these chemicals (which are peptide neurotransmitters) [68]. The release of cholecystokinin (CCK) (a hormone believed to mediate meal termination) is triggered by food consumption. This in turn activates CCK-A receptors in the pyloric region of the stomach [69]. The vagus nerve transmits this signal to the nucleus tractus solitarius in the brain stem. This signal is relayed to the hypothalamic region where integration with other signals occurs. Peptides such as enterostatin [70], neurotensin, and glucagon-like-peptide represent other potential peripheral satiety signals [71]. Any adverse effect of the test article on these chemicals will affect appetite/food consumption. Any changes in food consumption will in turn affect the body weight.

Body temperature: Body temperature and the immune system are closely related to each other during infections. Signals to the brain that control body temperature are sent during infections to elevate the temperature of the entire body, and this causes fever. No real infection exists during vaccination but the immune system may perceive one. The body learns how to fight off a real infection during vaccination. Body temperature should be measured at 6, 24, 48, and 72 h after each dose.

Injection-site evaluation: Draize scoring could be used for injection-site evaluation. It should include evaluation of edema, erythema, and eschar formation. The site of injection should be evaluated predosing, and at 24, 48, and 72 h post dosing. Inflammatory skin reactions should be graded according to the Draize (or modified) scales [72].

Ophthalmologic examination: Eyes are normally examined predosing and during the week prior to scheduled necropsy. The exam could include observation of the internal and external structures of the eye, such as the cornea, lens, and other transplant media (aqueous and vitreous humor), fundus including blood vascular, and optic disc.

The ophthalmologic examination could be (eg, uveitis[4]) indicative of inflammation in the eyes as reported in some vaccines.

Clinical chemistry: Blood samples for clinical chemistry evaluations could be collected in lithium heparin tubes for plasma. Clinical chemistry tests are used to diagnose disease, to monitor disease progression or response to therapy or toxin exposure, and to screen for the presence of underlying disease in apparently healthy animals. A wide variety of clinical chemistry tests are used for this purpose. The results of the following parameters are included in the clinical chemistry testing [73]:

1. Electrolyte balance (calcium, chloride, phosphorus, potassium, and sodium). Changes in free water and changes in electrolytes themselves (rate of intake, excretion/loss, and translocation within the body) affect the electrolyte levels in blood. As electrolytes are essential to the proper functioning of cells, the body maintains electrolyte concentrations within narrow limits.
2. Carbohydrate metabolism (glucose (principal source of energy for mammalian cells)). Sources of glucose include digestion of dietary carbohydrates, break down of glycogen in the liver (glycogenolysis), and production of glucose from amino-acid precursors in the liver (gluconeogenesis). Hormones (eg, insulin, glucagon, catecholamine, growth hormone, and corticosteroids) affect blood glucose concentration by facilitating its entry into or removal from the circulation. Changes in blood glucose levels due to test-article treatment could be an indication of an adverse event through the above-mentioned pathways.
3. Liver function (alanine animotransferase (ALT), aspartate aminotransferase (AST), sorbitol dehydrogenase (SDH), glutamate dehydrogenase (GLDH), total bile acids, alkaline phosphate (ALP), gamma-glutamyl transferase (GGT), and total bilirubin). Injury to liver parenchymal cells can be detected by measuring the hepatocellular leakage enzymes (ALT, AST, SDH, and GLDH). Enzyme leakage from cells through damaged cell membranes is indicated by the increased serum activity of these enzymes. Cholestasis, which implies impairment of bile flow, is diagnosed by the changes in ALP and GGT levels. Cholestasis will result in elevations of bilirubin in blood if it is severe. The main value of these enzymes is their greater sensitivity for this abnormality as compared to serum bilirubin levels alone. Gamma-glutamyl transferase is more specific than alkaline phosphatase for this purpose.
4. Muscle enzymes (AST (used also as liver injury marker, see above), creatine kinase, and lactate dehydrogenase). Creatine kinase, present in high concentration in the cytoplasm of myocytes, is the most widely used enzyme for evaluation of neuromuscular disease and is a "leakage" enzyme. This enzyme functions by making ATP available for contraction in muscles. This is done by the phosphorylation of ADP from creatine phosphate by catalyzing the reversible phosphorylation of creatine by ATP to form phosphocreatine + ADP. Phosphocreatine is the major storage form of high-energy phosphate in muscle. Lactate dehydrogenase is an enzyme that catalyzes the conversion of lactate to pyruvate. It is not tissue-specific, being found in a variety of tissues, including liver, heart, and skeletal muscle. Lactate dehydrogenase levels could be elevated by exercise, liver disease, muscle disease, and neoplasia.
5. Kidney function (creatine and blood urea nitrogen). Urea and creatinine tests are normally used as indicators of glomerular filtration rate (GFR). Ammonia, generated by catabolism of amino acids derived either from digestion of proteins in the intestines or from endogenous tissue proteins, is used by hepatocytes to synthesize urea. Urea is excreted by the kidney and intestine and in saliva and sweat. Plasma urea nitrogen concentrations depend on hepatic urea production and renal tubular flow rate. Increases in protein catabolism and digestion and decreases in GFR cause an increase in urea nitrogen levels. Plasma urea nitrogen levels are decreased when protein intake is decreased, protein anabolism, increase in excretion, and decrease in production (eg, liver disease). Muscle metabolism results in the production of creatinine. An energy-storing molecule in muscle called phosphocreatine undergoes spontaneous cyclization to form creatine and inorganic phosphorous. Creatinine is the result of creatine decomposition.
6. Proteins (total protein, albumin, globulin, and A:G ratio). Total protein and albumin are the measured parameters, and globulins and A:G ratio are calculated from them. Quantitative values for the above major proteins are the test results. However, there are many different types of proteins within the globulin fraction besides immune globulins, such as those associated with the acute-phase response, and this measurement does not provide information

[4] The middle layer of the eye is called uvea, which provides most of the blood supply to the retina. Uveitis is swelling and irritation of the uvea and could be caused by autoimmune disorders such as rheumatoid arthritis or ankylosing spondylitis, infection, or exposure to toxins. [Pubmed health (http://www.ncbi.nlm.nih.gov/pubmedhealth/PMH0002000/).]

on these fractions. Both quantitative and qualitative data for the different fractions that comprise total protein could be obtained by electrophoresis. Electrophoresis can be used on serum, urine, or body-cavity fluid samples (eg, cerebrospinal fluid).

7. Lipids (triglycerides, cholesterol). In serum, triglycerides are incorporated into lipoproteins that are composed of a coat of phospholipid, cholesterol, and proteins (apolipoproteins) enclosing a hydrophobic center of cholesterol esters and triglycerides. The most commonly occurring steroid is cholesterol. Cholesterol is a precursor of cholesterol esters, bile acids, and steroid hormones. It is derived from dietary sources and synthesized in vivo from acetyl-CoA in the liver (main site) and other tissues (intestines, adrenal glands, and reproductive organs).

Hematology: Blood samples for hematology evaluation could be collected in tubes containing EDTA (ethylenediaminetetraacetate). Blood samples for fibrinogen, prothrombin time, and activated partial-thromboplastin time could be collected in tubes containing sodium citrate.

The following parameters are included in hematology testing [74]:

(a) Red blood cells (hematocrit, hemoglobin, mean corp. Hb, mean corp. Hb. Conc., mean corp. volume, total erythrocyte count, and reticulocytes). Hematocrit (HCT) is calculated as the product of the mean cell volume (MCV) and the red blood cell (RBC) count. Packed cell volume (PCV) is a directly measured value obtained from centrifuging blood in a microhematocrit tube in a microhematocrit centrifuge. Hemoglobin concentration (Hb) is reported as grams of hemoglobin per deciliter of blood (g/dL). Hemoglobin concentration of whole blood normally is about one third of the HCT (ie, the MCHC is 33%) because red cells are approximately 33% hemoglobin. The MCV, expressed in femtoliters (fL; 10^{-15} L), indicates the volume of the "average" red cell in a sample. Mean cell hemoglobin (MCH) represents the absolute amount of hemoglobin in the average red cell in a sample and its units are picograms (pg) per cell. The MCH is calculated from the Hb and the RBC values using the following equation: MCH (pg) = (Hb × 10) ÷ RBC. The MCHC is the mean cell hemoglobin concentration, expressed in g/dL. It is calculated from the Hb and the PCV using mean corpuscular hemoglobin concentration (MCHC) = (Hb ÷ PCV) × 100. The term "hypochromic" is used for red cell populations with values below the reference interval. This can occur in a strongly regenerative anemia, where an increased population of reticulocytes with low Hb content "pulls" the average value down. Low MCHC can also occur in iron deficiency anemia, where microcytic, hypochromic red cells are produced because of the lack of iron to support hemoglobin synthesis.

Reticulocytes, which are released in increased numbers into the blood from bone marrow as a response to anemia, are young, anucleate erythrocytes. Hemolysis (destruction) or loss (hemorrhage) of erythrocytes, in most species, is the cause of anemia. To determine whether the bone marrow is responding to an anemia (given sufficient time) by increasing red blood cell production, identification of immature anucleate red blood cells is required. This is termed a regenerative response. Detecting immature erythrocytes by virtue of the presence of RNA in the form of ribosomes and rough endoplasmic reticulum in their cytoplasm is required to evaluate the bone marrow response. The more immature the cell, the more RNA it contains. In contrast, mature red blood cells, which are no longer synthesizing hemoglobin, contain very small amounts or no RNA.

(b) White blood cells [(WBCs) basophils, eosinophils, lymphocyte, macrophage/monocyte, neutrophil, leukocytes, and large unstained cells]. The WBC (thousands/μL), total number of leukocytes, is a count of nuclei or total nucleated cell count. If nucleated red blood cells (nRBCs) are circulating in blood, they will be included in the nucleated cell count. The WBC, in this case, represents the leukocyte count only after it has been corrected for the nucleated red cells (nRBCs). The correction is made as follows: corrected WBC = nucleated cell count × (100 ÷ [nRBC + 100]).

(c) Clotting parameter (mean platelet volume, fibrinogen, prothrombin time, and activated partial-thromboplastin time). Platelets play a fundamental role in hemostasis (formation of blood clots) and are a natural source of growth factors. A subjective estimation of platelet numbers could be made during examination of the stained blood film by plate smear. The size and number of platelet clumps is included in this estimation. Fibrinogen (*factor I*) [75] is synthesized by the liver and is soluble plasma glycoprotein that is converted by thrombin into fibrin during blood coagulation. To form a clot, fibrin is then cross-linked by factor XIII. It has been shown, in recent research, that fibrin plays a key role in the inflammatory response and development of rheumatoid arthritis. Prothrombin time is a blood test that measures the time it takes for plasma to clot, to check for bleeding problems, or to check whether medicine to prevent blood clots is working. Activated partial-thromboplastin time is used to detect abnormalities in blood clotting [76] and to monitor the effectiveness of heparin treatment.

Urinalysis: Urinalysis is the physical, chemical, and microscopic examination of urine. It involves a number

of tests to detect and measure various compounds that pass through the urine. There is an array of tests performed on urine and one of the most common methods of medical diagnosis [77]. Urine samples will be tested for the following:

1. Physical color and appearance: What does the urine look like to the naked eye? Is it clear or cloudy?
2. Is it pale or dark yellow or another color?
3. The urine-specific gravity test reveals how concentrated or dilute the urine is.
4. Microscopic appearance: The urine sample is examined under a microscope to look at cells, urine crystals, mucus, and other substances in the sample and to identify any bacteria or other germs that might be present.
5. Chemistry: A special stick ("dipstick") tests for various substances in the urine. The stick contains little pads of chemicals that change color when they come in contact with the substances of interest.

Bone-marrow smears: A bone-marrow sample is usually collected from the posterior iliac crest. Reasons to do a bone marrow biopsy include anemia of unknown cause, leukopenia, leukocytosis with immature granulocytes and/or blasts in the blood, and occurrence of unusual cells in blood (dwarf megakaryocytes, thrombocytopenia, and marked thrombocytosis).

C-reactive protein: C-reactive protein (CRP) is the primary acute-phase reactant in rabbits, monkeys, and humans. For these species, assays for CRP measurement are commercially available. When adequately sampled, CRP is indicative of a systemic inflammatory response that could be an indicator of potential toxicity [216]. This is particularly true when evidence of other toxicities, such as weight loss, are also found. Acute-phase reactants are a nonspecific inflammatory response and are not specifically associated with a particular type, variety, or class of injury (eg, liver or renal harm). When CRP is measured in rabbits or monkeys, there is no need to run serum electrophoresis analysis because adequate information on acute-phase reactions will be generated from the CRP data.

C-reactive protein is not the primary acute-phase reactant in rodents (rat or mouse); however, α1-acidic glycoprotein and α2-macrogobulin are responsive, inflammatory markers. Hence, although there is no need to measure CRP when rodents are proposed for use in a study, the equivalent, responsive acute-phase reactants should be measured. Alternatively, rodent acute-phase reactants may be measured by plasma electrophoresis since they occur in a fractionated part of the different globulins.

Creatine kinase (also known as creatine phosphokinase or phospho-creatine kinase): This is an enzyme expressed by various tissues and cell types. An inflammatory response to intramuscular injection of the vaccine might cause some minimal muscle degeneration, which may be reflected in creatine kinase levels. This inflammatory response is considered part of the expected mechanism of toxicity due to the means of vaccine administration.

Clinically, creatine kinase is assayed in blood tests as a marker of myocardial infarction (heart attack), rhabdomyolysis (severe muscle breakdown), muscular dystrophy, the autoimmune myositides, and in acute renal failure.

Antibody analysis (serology): It is critical to measure the immune responses for any vaccine and/or adjuvant and this is recommended in the WHO guideline [64]. The homeostatic condition in which the body maintains protection from infectious disease is called immunity. Immunity allows an individual to distinguish foreign material from "self" and neutralize and/or eliminate the foreign matter through a series of delicately balanced, complex, multicellular, and physiological mechanisms [78]. Promoting the cellular and/or the humoral immune responses are the primary purpose of vaccine developments. Serology data help in demonstrating the exposure to the vaccine, confirms the relevance of the animal model for evaluating the potential toxicity of the vaccine, and might allow the correlation between a toxic effect and the immune response induced [79]. ELISA (enzyme-linked immunosorbent assay) and other methods are used to measure specific antibody responses (humoral arm of the immune response). In the meantime, assays measuring cytokine-secreting antigen-specific T lymphocytes such as γ-interferon ELIspot [80] are used to evaluate the cellular arm of the immune response.

Necropsy: Animals are normally euthanized at different time points, depending on the study design and the expected responses to the test article under investigation. Terminal animals are usually necropsied a few days (eg, 2–7 days) after the last treatment, which helps in investigating the early effects after vaccination. Recovery animals are normally used to detect any delayed toxicity and/or to determine whether any earlier detected effects (if any) have resolved over time. Normally the number of animals in both terminal and recovery groups per sex are the same.

Histopathological evaluation: Gross examinations should be conducted on all major organs, and microscopic evaluation should be conducted on a complete list of tissues [64]. The site of vaccine injection (quadriceps and skin over the quadriceps for intramuscular (IM) injection) should be examined carefully. Brain, kidneys, liver, and reproductive organs are considered pivotal, and should be evaluated for any adverse changes. Immune organs

such as spleen, thymus, and draining lymph nodes are evaluated for any changes that might indicate a positive and/or negative response. The seriousness of the histopathological findings in some cases depends on other findings (eg, clinical pathology results). For example, vacuolation in the liver can be a normal finding, or may be indicative of toxicity. Vacuolation when accompanied by increases in clinical chemistry parameters such as liver enzymes (which in themselves would be of concern) is considered an indication of toxicity. However, vacuolation is considered an adaptive response when it occurs without other accompanying changes. For instance, metabolic activation could lead to vacuolation in many cell types, and would not be accompanied by other changes indicative of frank toxicity.

Unless they are severe, the intended immunological and inflammatory responses to the vaccine are not considered adverse effects. In repeat-dose studies, inflammation at the site of injection, hyperplasia and hypertrophy of lymph nodes draining the injection site, increase in spleen weight, and clinical pathology changes (eg, increases in white blood cells, increases in serum globulin, and decreases in serum albumin) are considered the intended immunological and inflammatory responses.

Reproductive and Developmental Toxicology Studies

Reproductive and developmental toxicology studies should be included in the IND package if the vaccine under study is intended to be administered to women of childbearing potential. This is also the case if the vaccine is specifically designed for maternal immunization to prevent infectious disease in the neonate by the passive transfer of antibodies (eg, the vaccine against group B *streptococcus*, which can be life threatening during the neonatal period) [81]. There are exceptions, as certain vaccines may automatically be contraindicated for pregnant women or to those planning to become pregnant [82]. For example, the smallpox vaccine is contraindicated for women who are pregnant, and women who plan to become pregnant within 4 weeks of vaccination. In addition, pregnant women are advised to avoid close contact with persons recently vaccinated, as in the case of rubella [83].

Studying the potential effects of the vaccine on fertility, fetal development, and postnatal development of the offspring is critical [84]. Sexual organs and their functions, endocrine regulation, fertilization, transport of the fertilized ovum, implantation, and development could all be affected by toxic effects of the vaccine [85]. Abnormal development of the fertilized egg through the embryo, fetus, and the offspring all the way to maturity, due to test-vaccine exposure, is a subset of reproductive toxicology called developmental toxicology. Developmental studies include the studies of the prenatal (embryonic and fetal) and postnatal (development following birth until the end differentiation of organs is achieved) events.

Choice of species depends on vaccine immunogenicity, and on the relative rate and timing of the placental transfer of antibodies. For example, in rats and mice, 90% of antibodies are transferred (postnatally) in milk. However, the majority of antibody transfer in rabbits occurs across placenta (prenatal).

Reproductive studies should be designed following ICH S5(R2) guidelines [86]. One species is required for this kind of study. Animals should be immunized a few weeks before mating and boosted immediately prior to mating (Fig. 27.1). One subset of pregnant females (20/group) should be submitted to cesarean section and fetal examination on gestation day (GD) 18 for mice and on GD 20 for rats. Another subset (20/group) should be allowed to litter, and the postnatal development (PND) of the pups should be followed up to weaning (rodent – PND 21). To assess the potential for long-lasting, permanent changes, the study could be extended to include assessment of the immune system (developmental immunotoxicity testing) in the offspring at 6–8 weeks.

Serum antibody levels should be determined as follows:

- F0 females: At predose, end of gestation and lactation periods.
- F1 fetus: Cord blood.
- F1 pup: Postnatal day 21.

Additional assessments can be conducted. Histochemical analysis for antibody deposition could be conducted if the vaccine induced adverse effects. Neurological assessments and immunological endpoints could be also included.

CBER guidelines[5,6] indicate that subjects may be included in clinical trials without developmental toxicity studies, provided appropriate precautions are taken to avoid vaccination during pregnancy. Developmental toxicity study reports can then be supplied with the biologics license application submission. Depending on the available toxicology information from the preclinical and the clinical studies, test articles are assigned different pregnancy categories. The FDA has assigned the following pregnancy categories.[7] Pregnancy and lactation labeling

[5] http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm076611.htm.
[6] http://www.fda.gov/OHRMS/DOCKETS/98fr/992079gd.pdf.
[7] http://www.scribd.com/doc/2278291/FDA-Pregnancy-Categories.

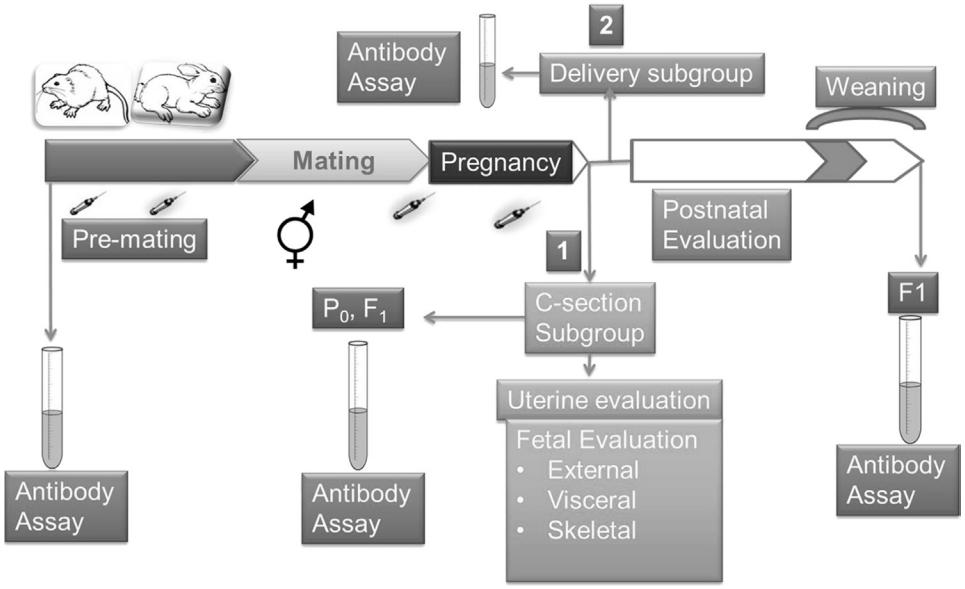

FIGURE 27.1 Schematic representation of Development Toxicology Study Design for vaccines. *Figure courtesy of Ali S. Faqi from MPI Research.*

rules published on December 4, 2014 are to replace the pregnancy categories below. The new rules are to:

1. Amend the physician labeling rule.
2. All prescription drugs approved on or after June 30, 2001 must revise content and format of the pregnancy and nursing mothers (lactation) subsections of labeling (pregnancy letter categories are replaced with an integrated risk summary).
3. All prescription drugs are required to remove pregnancy letter categories.
4. Staggered implementation over 3–5 years.

The required labeling elements are:

1. Pregnancy exposure registry
2. Risk summary that includes:
   a. Risk statement based on human data (if available)
   b. Risk statement based on animal data (summary of the available animal data, a statement if studies do not meet current standards, or a statement for no data existence)
   c. Risk statement based on pharmacology (statement regarding the mechanism of action (MOA) and potential associated risks when the drug has a well-understood MOA)
   d. Background risk information in general population (background risk for major birth defects and miscarriage in the US general population)
   e. Background risk information in disease population (background risk for major birth defects and miscarriage in the US diseased population)

Previously, the FDA had assigned the following pregnancy categories,[7] which are no longer applicable:

Category A

Adequate and well-controlled studies have failed to demonstrate a risk to the fetus in the first trimester of pregnancy (and there is no evidence of risk in later trimesters) in women.

Category B

Animal reproduction studies have failed to demonstrate a risk to the fetus and there are no adequate and well-controlled studies in pregnant women.

Category C

Animal reproduction studies have shown an adverse effect on the fetus and there are no adequate and well-controlled studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks.

Category D

There is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience or studies in humans, but potential benefits may warrant use of the drug in pregnant women despite potential risks.

Category X

Studies in animals or humans have demonstrated fetal abnormalities and/or there is positive evidence of human fetal risk based on adverse reaction data from investigational or marketing experience, and the risks

involved in use of the drug in pregnant women clearly outweigh potential benefits.

Mutagenicity Studies

Generally, mutagenicity studies are not required for vaccines (WHO guidelines on nonclinical evaluation of vaccine [64] and European Medicines Evaluation Agency (EMEA) [63]). Genotoxicity studies might not be relevant for adjuvant of biological origin [87]. The potential for gene mutation, chromosome aberrations, and primary DNA damage might be needed for synthetic adjuvants, because they are considered to be new chemical entities [88].

Carcinogenicity Studies

Generally, carcinogenicity studies are not required for vaccines (WHO guidelines on nonclinical evaluation of vaccine [64] and EMEA [63]). This is because of the low dose and the low usage of the adjuvants, meaning that the risk of tumor induction is very small, according to EMEA guidelines [89].

Safety Pharmacology Studies

These studies are performed to evaluate the adverse effects of the test article on physiological functions such as those of the cardiovascular system, respiratory system, and central nervous system [90]. Central nervous system studies include the evaluation of motor activity, behavioral changes, coordination, sensory/motor reflex responses, and body temperature. Cardiovascular system evaluation includes blood pressure, heart rate, and electrocardiogram measurements. In vivo, in vitro, and/or ex vivo evaluations, including methods for repolarization and conductance abnormalities, should also be considered. Respiratory rate, tidal volume, or hemoglobin oxygen saturation should be measured as part of the respiratory system evaluation. For vaccines, separate safety pharmacology studies are not performed [91]. These studies, which evaluate body temperature, electrocardiogram, and the central nervous system, could be included in the repeat-dose toxicity study if needed [64]. For more details about the safety pharmacology studies, refer to "Guidance for Industry S7A. Safety Pharmacology Studies for Human Pharmaceuticals" [90].

Other Toxicity Studies

For certain types of vaccine, specialized toxicity studies are needed. For new, live attenuated virus vaccines that have either a theoretical or an established potential for reversion of attenuation [92] or neurotropic activity [64], virulence and neurovirulence studies are needed. Polio and yellow fever vaccines fall into this category. This is based on the detailed knowledge of their neurotropic behavior. A neurovirulence test for a polio vaccine is part of routine batch-release procedures, and for yellow fever vaccine is designed to allow quantitative assessment of the effects of the virus by examination of specific areas following directed inoculation.

Vaccines with good safety records, such as measles, mumps, and varicella viruses, do not require reevaluation by neurovirulence tests when there are minimal changes to seed lots or to manufacture [92].

Since the early 1990s, a new approach to vaccination has been actively developed. These novel approaches include the direct introduction of plasmid DNA containing the gene encoding the antigen against which an immune response is sought by incorporating antigens into appropriate host tissues and the in situ production of the target antigen(s). The advantages of this approach over traditional approaches are that it stimulates both B- and T-cell responses, the vaccine has improved stability, the absence of any infectious agents, and the relative ease of large-scale manufacture [93]. Vaccines are generally used as biological medicinal products for the prophylaxis of infectious disease, but DNA vaccines are also being developed for therapeutic use (eg, against infectious disease or other diseases such as cancer). Using genes from a variety of infectious agents, including influenza virus, hepatitis B virus, human immunodeficiency virus, rabies virus, lymphocytic choriomeningitis virus, West Nile virus, malaria, and *mycoplasma*, many scientific publications [93] explore the potential of DNA vaccination and immune responses in animals. For nucleic acid and viral vector-based vaccines, biodistribution studies are necessary to determine the tissue distribution following administration and the potential for the vector to integrate into the host genome [93,94]. The design of nonclinical safety tests should take into consideration the use of the DNA vaccine and the risk/benefit situation. In addition to following GLP requirements for preclinical toxicology studies (see above), DNA studies should also evaluate any local inflammatory response (eg, myositis), organ-specific autoimmunity, immunopathology, and other relevant parameters. In particular, where the encoded antigen is a self-antigen, or may show self-antigen mimicry, a wider range of studies (including autoantibodies) may be necessary to address the specific concerns [93].

ANIMAL MODELS FOR VACCINE RESEARCH

Pasteur investigated anthrax, *Pasteurella multocida*, and rabies pathogenesis in animal models [95]. He confirmed that different species could be infected by certain pathogens. He also confirmed that an old culture of *P. multocida* (chicken cholera) kept in the laboratory without passage could protect chickens against virulent *P. multocida* challenge [95]. The concept of vaccinating dogs against rabies was also discovered by Pasteur [96]. Other

examples of animal usage in vaccine research include the use of virus-like particles (VLPs) for immunization against papillomavirus [97]. To control the disease caused by bovine, canine, and rabbit papillomavirus, recombinant papillomavirus VLPs was used [98,99]. This provided the basis for subsequent licensure of a bivalent and quadrivalent human papillomavirus (HPV) vaccine to prevent cervical cancer [100,101]. It has been confirmed through the development of this vaccine that studies in animals remain relevant to the control of infectious diseases in humans. Animal models in human vaccine development have different applications, such as:

1. Route of infection, transmission of disease, and analysis of disease pathogenesis
2. Host immune responses to natural infection and vaccination characterization
3. Onset and duration of vaccine-induced immunity assessment
4. Mucosal versus systemic immunity induction
5. Novel strategies for vaccine delivery and formulation development.
6. Clinical symptoms and disease transmission following infection reduction
7. Novel vaccination concepts (such as in utero or maternal immunization) development

Vaccine parameters requiring the consideration of animal models are [102]:

1. Vaccine safety
2. Duration and onset of immunity
3. Mucosal, maternal, and neonatal vaccination
4. Novel vaccine technologies
5. Vaccination of the elderly
6. Therapeutic vaccines for noninfectious diseases

Whether the study is intended to study toxicology or measure the efficacy of a new vaccine, selecting the right animal model is critical. For instance, a limited number of hosts including nonhuman primates, germ-free or barrier raised piglets, germ-free dogs and cats will be colonized by *Helicobacter pylori*. Investigators prefer working with small animals to larger animals. For example, the ferret has been successfully used to investigate gastritis and antimicrobial agents and *Helicobacter felis* mice have been used as an animal model for the study of *H. pylori* [103].

The ability to reproduce aspects relevant to human physiology is the hallmark of an appropriate animal model and its utility for vaccine development [59]. Humans or animals are ultimately the target population for the vaccine. Good models should share the same physiological characteristics (ie, humans and pigs share the same physiology of the skin), or at least reflect them as closely as possible. Ethical use of animals in human vaccine research requires the selection of those that match the human disease as closely as possible. The overall number of animals used for biomedical research will be reduced according to this criterion.

Anatomical, physiological, and immune system differences between species influence their relative responses. As part of the effort to find and develop new vaccines or adjuvants, animal models are typically used to discriminate between various antigens and their combination with different adjuvants. These animal models are useful because they possess the biological complexity of the immune system that may be predictive of humans and potential adverse effects. Although models such as transgenic animals exist, which possess enhanced qualities to represent various aspects pertinent to modeling the human immune system, these are not commonly used for toxicity assessment at this time. Strain- and antigen-dependent immunological responses will occur in both rats and mice [104]. These differences exist for both humoral and cell-mediated immunity [104].

For the host, criteria to consider when choosing animal models for vaccine development are similarities in:

1. Immune organ development
2. Transport of antibodies across the mucosal surfaces (surface IgA)
3. Route of transmission
4. Duration of immune memory
5. Pattern of pathogenesis
6. Receptors
7. The immune response ontogeny
8. Lifespan and duration of neonatal, adolescent, and adult period
9. Physiology (ie, skin) for specific delivery
10. Access to mucosal and systemic immune compartments
11. Transfer of passive immunity via the placenta, colostrum, and milk.

The pathogen criteria are similarities in:

1. Replication and spread of the pathogen
2. Virulence and pathogenesis
3. Route of entry of the pathogen in animal model
4. Genetic and antigenic characteristics.

To elucidate aspects of immune physiology in vivo, the mouse is an excellent animal model [2]. Although frequently used, it does have limitations in the study of the etiology of infection and disease pathogenesis. Murine models are suitable to study acute extracellular bacterial infections, but they are of limited value for the study of intracellular viral, bacterial, or parasitic infections [2]. The value of mice and rats as models to study most intracellular infections is limited because of the complex and unique etiology of intracellular infections and the narrow host range of infectivity of individual pathogens [2]. Exceptions to this general concept are a small

number of specific intracellular murine infections, like the one involving lymphocytic choriomeningitic virus infection [105], which has yielded unique insights into the understanding of protective immunity and intracellular infection.

Primates, guinea pigs, rabbits, cats, and ferrets have been used selectively as relevant models to study vaccination. In earlier studies on tuberculosis [106] (Tb) and more recently for simian immunodeficiency virus (SIV) studies [107] as a model for Tb and HIV in humans, primate models have been used. As an experimental infection model to evaluate human tuberculosis vaccines, guinea pigs have been used [108]. Guinea pigs are inordinately susceptible to tuberculosis following infection with *Mycobacterium tuberculosis* or *M. bovis*. While potentially useful when studying pathogenesis, this may limit the value of the guinea pig as a model to study Tb protective immunity. Because they produce tubercles and granulomatous disease (similar to that found in domestic livestock and humans), guinea pigs have been used extensively in tuberculosis research.

Orme et al. [109] reported the advantages and disadvantages of a range of animal models of tuberculosis as shown in Table 27.3.

Influenza Vaccines and the Selection of the Appropriate Animal Model

Because human influenza virus isolates replicate in both the upper and lower respiratory tracts with clinical signs of disease at reasonable virus doses in ferrets, they were used in studies to support the safety of live influenza vaccines [110]. However, because antigen-specific CD8+ T cells can easily be measured in the lymph nodes, circulation, and lungs of the mouse, this animal model is suitable to generate data to support the potential immunogenicity of a novel vaccine targeting the induction of these effector cells [110].

Mice, Cotton Rats and Guinea Pigs

To understand the mechanisms of the immune protection and the contributions of IgA, IgG, CD4+, and CD8+ T cells to immunity, mice were used on regular basis [111]. Mice have been used to evaluate the pathogenesis of avian influenza viruses and the 2009H1N1 virus and to examine the protective activity of vaccines against these strains [112–115]. As in any animal model, the above-mentioned advantages of using mice in influenza studies are offset by their disadvantages. One of these is that the mice are not a natural host of the influenza virus. Thus to determine vaccine effectiveness, studies are usually performed with virus strains that have been adapted to replicate in mice [116]. Alternatively, large inocula are administered directly to the lower respiratory tract to induce disease.

Prior to using this model for testing novel vaccines, differences between mouse and human innate and adaptive immunologic interactions should be considered [117]. For RSV infection, the BALB/c mouse was used because it mimics human respiratory disease [50,54].

Cotton rats have also been used for studies of immunity and viral pathogenesis [118]. Clinical signs of infection are evident after intranasal inoculation with reasonable virus doses in this model [119]. In addition, in this model, respiratory rate as a measure of influenza virus-induced disease is helpful in providing a relevant endpoint to evaluate disease in live animals over an extended period of time [120]. The cotton rat was also a good model for evaluating the impact of the early innate response on immunity [121].

Mice and cotton rats are not good models for evaluating the spread of infection between animals, because influenza viruses are not transmissible between them.

TABLE 27.3 Advantages and Disadvantages of Animal Models of Tuberculosis

Species	Advantages	Disadvantages
Mouse	Inexpensive Extensive immunological database Inbred strains show considerable range of resistance or susceptibility to aerosol infection importance and role of T-cell subsets consistent with observations in humans	Delayed type hypersensitivity response is poor Hard to measure convincingly Takes months to develop necrotic pathology in lungs Small window of protection in vaccine studies (1.0–1.25 log)
Guinea pig	Progression of disease very similar to humans Large window of protection in vaccine studies (2–3 log)	Expensive; requires large aerosol chamber and extensive P3 facilities Limited immunological reagents
Rabbit	Models “extremes” of disease such as liquefied cavities and miliary disease Quick; develops severe disease in 6–8 weeks	Expensive; requires extensive P3 facilities Animal husbandry issues (eg, sheds bacteria in urine) Limited immunological reagents
Monkey	Similarity to humans Probably required for regulatory approval before widespread human testing	Expensive Requires extensive P3 facilities Aggressive (can bite) Might carry pathogenic viruses General public opposes use

The guinea pig is a good model to be used in such studies [122,123] because this animal model supports influenza virus replication in its upper and lower respiratory tracts [124]. In addition, the guinea pig provides a means of comparing the effectiveness of influenza vaccines by showing the differences in protection after immunization with inactivated and live, attenuated vaccines [125]. However, because a correlation between this endpoint in an animal model and infection or disease rate in humans has not been demonstrated, the real value of determining the impact of vaccination on virus transmission in guinea pigs is questionable [110].

Ferrets

Ferrets have been used for influenza virus studies since 1933. In experiments, these animals showed sign of disease following inoculation with filtered nasal secretions from an individual with respiratory symptoms [126]. Human H5N1 are highly pathogenic in ferrets, inducing sneezing, coughing, fever, weight loss, diarrhea, and neurological signs [110]. Not only could the virus replicate in the nasal turbinates and lungs, H5N1 can spread to the brain, spleen, and intestine in these animals [127]. Therefore ferrets are commonly used to evaluate the immunogenicity and effectiveness of pandemic influenza vaccines. For the advantages and disadvantage of this animal model when studying the influenza virus, Eichelberger and Green [110] should be consulted.

Pigs and Cats

There was renewed interest in the pig as a model for studies of influenza viruses after the emergence of the pandemic swine-origin H1N1 strain in 2009 [128]. Nasal discharge, cough, fever, labored breathing, and weight loss have been reported in pigs as the cause of certain swine strains, which made this animal a useful model for studies of immunity and pathogenesis [129–131]. For study of the 2009H1N1 pandemic virus (A/California/04/09), specific-pathogen-free miniature pigs were used [132]. This model was particularly attractive because of the availability of a number of reagents enabling studies of immune correlates of protection [133]. Highly pathogenic avian viruses did not cause severe clinical signs of disease in pigs [134]. However, highly pathogenic H5N1 viruses did cause disease and death in cats [135,136]. Thus cats were considered a good model to test protection against disease and death due to highly pathogenic avian-origin strains. H1N1 virus causes a moderate level of disease in cats when infected intratracheally, replicating primarily in the lungs but can also be isolated from other organs [137]. Pigs and cats are not extensively used for studies of pathogenesis, immunity, or transmission of human influenza and therefore are not currently used routinely to support human vaccine studies [110].

Monkeys

Cynomolgus, rhesus, and pigtailed macaques have been used for influenza virus infection [138–140]. The cynomolgus macaque has been designated as a good model for the study of the H1N1 virus of 1918. This type of monkey showed an atypical innate immune response correlating with lethal disease [141]. It has also been used to evaluate the effectiveness of a recombinant modified vaccinia Ankara virus expressing HA against highly pathogenic H5N1 infections [142].

In general, to study influenza [143] and distemper [144] infections, ferrets have been used.

The Selection of Animal Models for Other Vaccines

Because the immunological cells cannot be transferred between histoincompatible outbred individuals, the use of the outbred animals is limited. Cats are used to study feline immunodeficiency virus (FIV), which is the analog of HIV infection in humans [145], whereas rabbits have been used to study immunity to a variety of toxinogenic bacterial infections, which require neutralizing antibodies as the main pathway for protection [146].

Guinea pigs were the first model to be used for *Leishmania enrietti* infection. T-cell responses to parasite antigens develop within 2 weeks of infection, and the lesions heal within ~10 weeks in guinea pigs [147]. Infection of inbred mice with *Leishmania* species pathogenic for humans superseded the *L. enriettii* guinea pig model [148]. The spectrum of disease manifestations observed in human leishmaniasis can be mimicked in the laboratory by infection of different inbred strains of mice with *L. major*. Including a range of susceptibility states depending on the strain of mouse used, the mouse model reproduces many aspects of human disease. Upon infection, BALB/c mice develop large skin ulcers, which expand and metastasize, leading to death. However, C57BL/6 and CBA/N mice are resistant; they develop small lesions that cure in 10–12 weeks, and are resistant to reinfection. Intermediate susceptibility was reported in most other strains of mice [149]. Both susceptible and resistant mice produce Th2 cytokines during the period of active lesion development [150,151]. The difference between susceptible and resistant mice is that the latter are able to switch to a Th1 profile and control the disease [152,153].

The golden hamster was one of the early animal models for the study of visceral leishmaniasis. Visceral disease and death is the result of infection with *L. donovani* in this model. The aspects of the human disease mimicked in the hamsters are anemia, hyperglobulinemia, and cachexia, making it a useful tool for the characterization of molecules and mechanisms involved in

pathogenesis [154]. Recently, the hamster has been used primarily as a source of *L. donovani* amastigotes, which seem to be the required lifecycle stage for infecting mice, the currently preferred model animal for visceral leishmaniasis. Inbred strains of mice display marked differences in susceptibility to infection with *L. donovani* [155]. The best animal model for visceral leishmaniasis is the dog, in which relevant immunological studies and vaccine development can be performed [156,157].

Other than humans, the only species susceptible to HIV-1 infection are the great apes, of which the chimpanzee has been used to study this virus. Limitations include scarcity of animals, cost, limited viral replication, and absence of disease when infected with patient isolates. The Asian macaque monkey has been used as a good model for SIV studies. The SIV-infected macaque has been used as a model for assessing HIV-1 vaccine strategies, because it develops an AIDS-like disease. Limitations include differences from HIV-1 in viral sequence and envelope epitopes. Recently, chimeric viruses have been constructed in the laboratory that express HIV-1 envelopes on an SIV backbone [158–160]. These constructed viruses are called simian/human immunodeficiency viruses (SHIVs). In macaques, the in vivo passage of these chimeric viruses resulted in SHIV induction of CD4+ lymphocyte loss, and death as a result of opportunistic infections [161].

Because genital disease in guinea pigs closely resembles that of humans [162], it has been used to test potential vaccines [163,164] and antiviral chemotherapies [165] for genital herpes. McClements et al. [166] reported that immunization with DNA-encoding herpes simplex virus type 2 full length glycoprotein D (HSV-2 gD) or a truncated form of HSV-2 g-induced immune responses in mice and protected them from lethal challenge with HSV-2. They also showed that a combination of these two DNAs protected guinea pigs from primary genital disease. McClements et al. [166] also found that protective immunity could be induced by low doses of DNA in the mouse model with only a single immunization. Additionally, other investigators demonstrated protective immunity in the mouse [167–169] and guinea pig [170], HSV-infection models were induced by multiple immunizations with higher doses of gD DNA or gB DNA. Provost et al. [171] reported that both the marmoset and the chimpanzee are useful models for hepatitis A virus behavior in man.

To select the right animal model for any vaccine development, safety and efficacy should be taken (equally) into consideration. A safe vaccine without good efficacy will be of no use and vice versa.

A good understanding of responses to vaccination in both neonates and the elderly is also required because they are at increased risk of contracting infectious disease. Studies in the mouse model have suggested that vaccine responses may be compromised in these age groups. The development of the murine immune system may not provide an appropriate model for evaluating immune responses in these two age groups. To evaluate vaccine immune responses in the neonate, and to address questions regarding possible interactions between vaccines and maternal antibodies, large-animal models may be much more appropriate [172–174]. However, other than mice, there have been very few investigations of vaccine responses in geriatric animals [175,176]. For the screening of adjuvants, the horse could provide geriatric populations [177].

An appropriate animal model is also needed for the development of mucosal vaccines. Disease protection against a wide variety of pathogens that invade through mucosal surfaces could be achieved by mucosal vaccination. Difficulties associated with efficient vaccine delivery and weak immune responses following mucosal immunization made the induction of protective immune responses at mucosal surfaces an elusive goal. Thus for the evaluation of mucosal vaccine-delivery technologies, effective and safe mucosal adjuvants, and the characterization of mucosal immune responses, an appropriate animal model is required. In mice, intranasal vaccination may be associated with inhalation and ingestion of vaccine antigens. This makes it difficult to discriminate between intranasal, oral, and intrapulmonary vaccination. However, larger animals like the pig or the cow can be used for the controlled delivery of vaccines to the nasal passages [178,179]. The nasal passages of these animals more closely resemble that of humans than do those of the mouse. Surgical models have also been useful for screening a variety of mucosal vaccine delivery technologies and potential mucosal adjuvants [180,181].

It is critical, when choosing an animal model, to ensure that the selected model simulates as closely as possible the events occurring in humans. The greater the similarity in patterns of pathogenesis between the two, the more likely it is that relevant correlates of immune-mediated protection will emanate from the model. The same route of exposure should be used. If the respiratory tract is the pathogens' route of entrance, then the aerosol challenge to expose the pathogen to the defenses of the upper respiratory tract should be used. Intratracheal challenge would not be considered appropriate, because it circumvents the various barriers of the upper respiratory tract. A similar pathogen dose to that which would occur naturally should be used. Use of excessive pathogen challenge, or an unnatural route of infection, might overcome the adaptive immune response. The structure, function, and development of the respiratory tract in the animal model should resemble that of humans when choosing a model for respiratory infections.

Because of the above-mentioned reasons, animal models will continue to play a critical role in human-vaccine

development, especially in the preclinical discovery phase. Thus it is critical to choose the most appropriate models and not restrict investigations to the least expensive and most convenient animal models. This will help make optimal use of animals and more rapidly bring safe and effective vaccines to the market.

ROUTES OF VACCINE ADMINISTRATION

Selection of an appropriate route for vaccine administration is a critical component of a successful immunization. Vaccines are normally administered by injection, either intravenous (IV), intramuscular (IM), or subcutaneous (SC) administration [182]. There are advantages and disadvantages for these routes of administrations. Vaccines could also be administered orally or intranasally, and these routes also have advantages and disadvantages, which will be discussed later in this section.

Intramuscular (IM) Vaccine Administration: The needle used to administer the vaccine to the muscle should be long enough to reach deep into the muscle. It should be inserted at a 90 degree angle to the skin with a quick thrust. It is not necessary to aspirate when using this route. A minimum of 1-inch separation is necessary when using multiple injections in the same extremity. The following vaccines should be administered by the intramuscular (IM) route: diphtheria–tetanus (DT, Td) with pertussis (DTaP, Tdap); Haemophilus influenzae type b (Hib); hepatitis A (HepA); hepatitis B (HepB); human papillomavirus (HPV); inactivated influenza (TIV); quadrivalent meningococcal conjugate (MCV4); and pneumococcal conjugate (PCV). Inactivated polio (IPV) and pneumococcal polysaccharide (PPSV23) could be administered either by IM or SC routes.

Subcutaneous (SC) Vaccine Administration: Subcutaneous tissue should be pinched up to prevent injection into muscle. The needle should be inserted at a 45 degree angle to the skin. It is not necessary to aspirate when using this route. A minimum of 1-inch separation is necessary when using multiple injections in the same extremity. The following vaccines should be administered by the SC route: measles, mumps, and rubella (MMR), varicella (VAR), meningococcal polysaccharide (MPSV4), and zoster (shingles (ZOS)).

To optimize the immunogenicity of the vaccine and minimize adverse reactions at the injection sites, most vaccines should be given via the intramuscular route into the deltoid or the anterolateral aspect of the thigh. Vaccine failure might be the result of injecting a vaccine into the layer of subcutaneous fat, where poor vascularity might result in slow mobilization and processing of antigen [183]. This might be the case in hepatitis B [184], rabies, and influenza vaccines [185]. Subcutaneous injection of hepatitis B vaccine leads to significant lower seroconversion rates and more rapid decay of antibody response when compared to intramuscular administration [183].

To initiate an immune response, the appropriate cells, eg, phagocytic or antigen-presenting cells, should be involved [186]. The layers of fat do not contain these cells, and when deposited in fat, the antigen may take longer to reach the circulation, potentially leading to a delay in processing by macrophages and eventual presentation to the T and B cells of the immune response. Antigens may also be denatured by enzymes if they remain in fat for hours or days. Thicker skin folds are associated with a lowered antibody response to vaccines [183,184].

Because adipose tissue has much poorer drainage channels than muscle, it retains injected material for longer periods, and is therefore more susceptible to its adverse effects [187]. Thus subcutaneous injections can cause abscesses and granulomas [183,187,188]. Because of its abundant blood supply, muscle tissue is probably spared the harmful effects of substances injected into it [187]. The antigen is adsorbed to an aluminum salt adjuvant in hepatitis A, hepatitis B, and diphtheria, tetanus, and pertussis vaccines, hence the intramuscular route is strongly preferred. Superficial administration of these vaccines may lead to an increased incidence of local reactions, such as irritation, inflammation, granuloma formation, or necrosis [184,189,190].

How deep a substance is injected is determined by the injection technique and needle size. A wide variation exists in thickness of the deltoid fat pad, with women having significantly more subcutaneous fat than men [183]. The use of longer needles might cause the patient more discomfort, but, because skeletal muscle has a poorer supply of pain fibers than skin and subcutaneous tissue, discomfort might be less [191]. Needle gauge is another important factor in vaccine administration [192], as the vaccine is dissipated over a wider area when using a wider bore needle. This reduces the risk of localized redness and swelling [193].

Intranasal Vaccines

Alternative routes of administration have been used to improve the protective immune responses at the very places in the body that certain viruses and bacteria are likely to target. Intranasal vaccines can induce protective immunity in the respiratory tract where the viruses attack.[8] By either slowing the rate of uptake of the antigens (eg, intranasal vaccines are taken into the body more slowly than injectable vaccines, thus reducing the risk of allergic reaction) or by administering the vaccine viruses to an area of the body that they do not typically grow in (thus reducing the disease-causing effects of some of the

[8] http://www.sciencedaily.com/releases/2011/04/110411194821.htm.

strains of live vaccine viruses), the side effects of the vaccine will be reduced. Intranasal administration is easy and acceptable to both humans and animals.

Avirulent intranasal vaccines could be given via the nostrils using special applicators. The cells lining the upper respiratory tract (nasal passages, throat, trachea) would then be coated by the vaccine and the virus would subsequently replicate in these cells. These viruses (and/or bacteria) will be attacked by the immune cells present in the respiratory tract, inducing a protective immune response that tends to remain within or near the respiratory tract.

If an animal received an intranasal vaccine, the lining of its respiratory tract would be coated with protective antibodies. Hundreds of memory cells, primed to recognize the antigens contained on the invading respiratory viruses, will be included in the regional, respiratory-system lymph nodes [194]. When the invading viruses and bacteria reach the respiratory tract, these antibodies and memory cells would react and eliminate them. This response is much more rapid than that produced by an injectable vaccine. This is because the resultant immune defenses are located in the same region as the invading pathogens. The invading viruses will not get the opportunity to damage many cells in this case. Moreover, clinical signs of disease should not occur or, if they do, they should be very mild.

There are advantages and disadvantages for intranasal vaccination [195]. The advantages are:

1. Improved patient compliance [196].
2. Improved penetration of (lipophilic) low molecular weight drugs through the nasal mucosa [197].
3. Due to large absorption surface and high vascularization, rapid absorption and fast onset of action is expected.
4. Avoidance of the gastrointestinal tract environmental conditions (chemical and enzymatic degradation of drugs) and the hepatic first-pass metabolism.
5. Direct delivery of vaccine to the lymphatic tissue [198].
6. Induction of a secretory immune response at distant mucosal site [198].
7. Because the uptake of viral antigen into the body is slower in intranasal vaccination, allergic reactions are less likely to happen.

The disadvantages are:

1. Mild upper respiratory tract infection could be induced. This is characterized by watery nasal and ocular discharge, sneezing, and even coughing. However, this is usually self limiting and very mild.
2. They are generally only effective against respiratory pathogens.
3. Intranasal vaccines needed every year.
4. Severe liver damage and even death of the animal could be caused by an accidental injection of the intranasal *Bordetella* vaccines [194].
5. Penetration to the brain through the olfactory region may be caused by nasally administered substances, including toxins and attenuated microorganisms.[9] For some vaccines and drugs targeting neurological diseases, such direct nose-to-brain transport may be advantageous but raises concerns about potential adverse effects when the brain is not the target organ.[8]

Alternative Routes

Few other noninjectable routes exist beside intranasal application. Orally and intraperitoneally administered vaccines (given into the abdominal cavity) have been investigated or approved for human use. These routes are used to improve the response of the gastrointestinal immune system to diseases like parvovirus and coronavirus. Polio vaccine, rotavirus, adeno, or typhoid are examples of orally administered vaccines. Dermal patches, sprays (vaccines applied to the skin surface), and transdermal vaccines (aerosolized vaccine particles that are forced at high pressure through the skin using special instruments, thus avoiding the need for needles) have been developed. DNA plasmid vaccines are typically administered by the IM or ID route and may be given by electroporation that propels DNA-coated gold particles into various tissues [199].

Vaccine Injection Versus Intranasal Administration (Live Attenuated Vaccines Versus Killed Vaccines) [200–202]

Killed (or subunit) vaccines do not replicate and stay in one spot for the immune system to "kill." Without virus replication, the immune system does not become exposed to the massive amounts of antigen generated by live viruses. Inefficient humoral immunity (fewer memory B cells and smaller amounts of antibody that don't last in the body as long) and cell-mediated immunity (not as many memory T cells waiting to target the next wild-type virus that comes along) will be developed. Humoral and cell-mediated immunity can be improved by:

1. Adding large quantities of killed virus or bacterial matter into each inactivated vaccine. The amount of antigen available for the immune system to recognize will be increased this way. However, this will increase the risk of allergic and local inflammatory injection site reactions.

[9] http://www.optinose.no/assets/documents/20030129173426_Nasal_delivery_of_vaccines.pdf.

2. Adding adjuvants to the vaccine designed to increase the effectiveness of the immune response. However, some adjuvants might increase the risk of allergic reactions, anaphylaxis, and injection site reactions. Most require a minimum of two doses to achieve the desired effect (risk of vaccine reaction with the second dose). They must be given by injection (not available by other routes of administration).

Live vaccines are more amplified and promote longer lasting humoral and cell-mediated immune responses, resulting in longer lasting, more rapidly induced immune protection. Because it replicates in the body, only a small amount of viral material needs to be injected. Less viral material means a reduced risk of allergic and injection site reactions. No adjuvant is required in this kind of vaccine, hence the risks of allergic and injection site reactions are reduced. Other than injection, live vaccines can be given by other routes (eg, intranasal). Thus live vaccines can potentially be tailored to induce immunity in the areas of the body where it will be most effective (eg, immunity in respiratory system to protect against respiratory viruses). The drawbacks of live vaccines are:

1. It must be stored carefully, or its potency may be lost.
2. Immunocompromised or pregnant animals/humans might get the disease.
3. Severe complications might be caused by certain live vaccines (eg, live rabies vaccines can cause fatal neurological disease in some dogs and cats).
4. Poorly produced vaccines may contain virulent organisms that could produce severe disease.
5. Some live vaccines can cause severe illness if given by the wrong route (eg, injectable cat flu vaccine viruses that accidentally get inhaled by a cat will produce marked signs of cat flu, and intranasal *Bordetella* vaccine viruses can cause liver damage and death if injected).

PRODUCT CHARACTERIZATION

In addition to toxicity studies, in vivo and in vitro assays play a significant role in assessing critical safety characteristics of vaccines. Testing encompasses assessments for identity, purity, safety, and efficacy in terms of antigenicity and potency. Generally, these types of study are aimed at detecting undesirable contaminants or impurities, characterizing the vaccine product, and ensuring conformation to specified manufacturing standards. Unlike toxicity studies, which explore the potential for unanticipated risk, or further refine the understanding of adverse effects, product characterization studies emphasize, quantify, and examine aspects that are associated with the properties of vaccines such as potency and are important to the consistent and safe manufacture of vaccines. In some respects, these studies may be considered to be focused toxicity studies that have restricted or narrow endpoints that include survival or clinical signs. Among the most important are tests for potency, general safety (21CFR610.11), neurovirulence (IABS Scientific Workshop on Neurovirulence Tests for Live Vaccines, WHO, 2005), tumorigenicity (Meeting Report, WHO Study Group on Cell Substrates for Production of Biologicals, WHO, 2007; European Pharmacopoeia Section 5.2.3), and pyrogenicity (21CFR610.13). The degree and nature of these tests depend on the immunological mechanisms involved in the action of the vaccine or the nature of potential unwanted constituents.

Vaccine potency tests typically measure the level of protection, either against a direct challenge using known quantities of infectious organisms, or more indirectly through exposure to serum containing neutralizing antibodies following incubation with a toxin. Determination of potency is generally made through a series of dilutions that are compared to standard references.

Unlike typical immunization protocols that utilize a prime and boost strategy of successive injections spaced over time, immunogenicity testing for product characterization is often limited to a single injection, because the initial response is believed to better discriminate the amount and quality of an immunogen.

The infrequent serious toxicities that have been associated with vaccines are often linked to the manufacturing process. Some early lots of the polio or "Salk" vaccine were not completely inactivated. This allowed contamination by live polioviruses and resulted in the paralysis of over 200 individuals [203,204]. Additionally, contamination of commercial vaccines, such as poliovirus, adenovirus [205,206], and yellow fever [204,207–210], during the 1940 and 1950s, demonstrated the potential for harm.

Among the different types of product characterization studies with toxicity-related endpoints are the following:

1. Insertional mutagenesis of DNA vaccines
2. Attenuation
3. Untoward immunization—hypersensitivity, autoimmunity, breaking immune tolerance

PEDIATRIC DRUG DEVELOPMENT (PRECLINICAL SAFETY EVALUATIONS)

Pediatric evaluations are required as part of new drug and biologics licensing applications in the United States and every marketing authorization application in Europe, unless a waiver has been granted [211]. It is advisable to acquire the approval of regulatory agencies (FDA and EMA) for any pediatric development plans before starting any pediatric clinical trials.

For juvenile toxicity studies (if pharmacological activity has been demonstrated), one species is acceptable [212–214]. The rat is the recommended species (if relevant), because it has developmental systems that can be easily monitored [211]. Other animal models could be used after careful consideration of its organ-system development relative to that of humans. Because species selection is limited by target specificity, the nonhuman primate (NHP) is the only suitable species for toxicity assessment [211]. The core requirement for preclinical testing of biopharmaceuticals is to establish pharmacological relevance in the test species [215]. Morford et al. [211] reported the advantages and disadvantages of species (NHP, rodents, dogs, and mini-pigs) for juvenile toxicity testing with biopharmaceuticals.

A number of documents developed by various regulatory agencies can provide supplementary information concerning various aspects of the topics discussed in this chapter:

1. WHO Guideline on Nonclinical Evaluation of Vaccines,
   - **a.** Annex 1, WHO Technical Report Series No. 927, 2005. This provides a good overall summary of both manufacturing and toxicity testing paradigms for vaccine products. Various sections cover a wide number of topics including toxicity study design, assessments as well as adjuvants and potency tests.
   - **b.** Annex 2, WHO Technical Report Series No. 987, 2014. This is an updated and more extensive guidance on the nonclinical and preclinical testing of adjuvants and adjuvanted vaccines. Due to the increased usage of novel adjuvants to enhance the immune responses of vaccines, this guidance should allow manufacturers and regulators to proceed in an efficient manner toward development and licensure of adjuvanted vaccines.
   - **c.** Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines, CPMP, EMEA, CPMP/465/95, 1997. This briefly describes a broader range of subjects in the nonclinical testing of vaccines.
   - **d.** Workshop on Nonclinical Safety Evaluation of Preventative Vaccines Recent Advances and Regulatory Considerations. The Society of Toxicology, Contemporary Concepts in Toxicology Section, US Department of Health and Human Services, Office of Women's Health, FDA, 2002. This is a transcript of a meeting between members of the FDA and various representatives from industry. Various perspectives and approaches to toxicity testing of vaccines are discussed.
   - **e.** Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications. CBER, FDA, US Department of Health and Human Services, 2010. This describes different product characterization studies including tumorigenicity and in vivo tests for adventitious agents.
   - **f.** Guidance for Industry: Considerations for Plasmid DNA Vaccines for Infectious Disease Indications. CBER, FDA, US Department of Health and Human Services, 2007. This contains a section on biodistribution as well as nonclinical tests for immunogenicity and safety regarding plasmid DNA vaccines.
   - **g.** Guidance for Industry: Consideration for Developmental Toxicity Studies for Preventive and Therapeutic Vaccines for Infectious Disease Indications. CBER, FDA, US Department of Health and Human Services, 2006. This provides information on timing and study design for nonclinical toxicity studies that target developmental and reproductive endpoints.

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